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ETHYLENE BIOSYNTHESIS BY ENZYME
SYSTEMS FROM BEAN COTYLEDONS

BY



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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

NOVEMBER, 1968

1968 (F)

71D

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Ethylene Biosynthesis by Enzyme Systems from Bean Cotyledons" submitted by Robert Anthony Stinson in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

The critical role that has been proposed for ethylene in fruit ripening and in a number of other physiological processes certainly justifies the intense research activities that have been directed towards the elucidation of a biosynthetic pathway by which the volatile is derived. The purpose of this dissertation research was to contribute to existing information on possible precursors and pathways for ethylene biosynthesis.

Changes in respiratory parameters (ADP/O ratios, respiratory control by ADP, rate of ATP hydrolysis, and respiration rate) of wax bean cotyledon mitochondria during germination were examined in relation to the pattern of ethylene evolution from a subcellular particulate fraction isolated from these cotyledons.

A technique for the preparation of a soluble enzyme system from a subcellular particulate fraction of bean cotyledons was developed. A 32,000 x g particulate fraction was rendered soluble by a 12 h treatment with 0.4% Triton X-100 and then subjected to gel filtration on Sephadex G-25. This enzyme system was used to study the conversions of the amino acids β -alanine and methionine, the aldehydes methional and propanal, and propionic acid to ethylene. Apparent nonenzymatic conversions of the latter three compounds did not allow an evaluation of their roles in enzymic reactions leading to ethylene. Evidence was obtained to support the proposal that β -alanine could be converted to ethylene through the intermediates malonic semialdehyde, β -hydroxypropionate, and acrylate, and that methionine could be converted to ethylene through the intermediate methional.

In support for the involvement of malonic semialdehyde in the derivation of ethylene from β -alanine, a transaminase was found that converted β -alanine to malonic semialdehyde. The enzyme would transaminate β -alanine with either oxaloacetate or pyruvate as amino acceptor, but not with α -ketoglutarate.

ACKNOWLEDGMENTS

The sincere interest and generosity of knowledge expressed by my Committee Chairwoman, Dr. Mary Spencer, was very much appreciated.

My wife, Gail, deserves special commendation, not only for bearing the charge that comes with being a femme couverte but also for the extra encouragement and the extra morale that she so willingly gave.

I am grateful, for financial support, to the National Research Council of Canada for a bursary and three studentships and to the University of Alberta for a Dissertation Fellowship.

Appreciation is extended to the numerous other individuals who contributed to the work reported in this thesis. Special thanks go to Mrs. Doreen Simpson for her dedicated typing of the manuscript.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ADP/O	$\mu\text{moles ADP phosphorylated} \div \mu\text{moles oxygen (O) consumed}$
α -KG	α -ketoglutarate
ATP	adenosine triphosphate
BSA	bovine serum albumin
c	curie (Ci)
CoA	Coenzyme A
cpm	counts per minute
dpm	disintegration per minute
DTT	dithiothreitol
EDTA	disodium ethylenediaminetetraacetate
GC	gas chromatograph
GRC	gas radiochromatograph
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
MSA	malonic semialdehyde
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
OAA	oxalacetate
Pi	inorganic phosphate
RC	respiratory control
TCA	trichloroacetic acid
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
TLC	thin-layer chromatography
TPP	thiamine pyrophosphate
Tricine	N-tris(hydroxymethyl)methylglycine
Tris	tris(hydroxymethyl)aminoethane
(U)	uniformly labelled

INTRODUCTION

The physiology and biochemistry of ethylene action have been studied for many years. The extreme diversity of effects of the volatile and its essentiality in fruit ripening have prompted many workers to investigate possible biosynthetic schemes. Penicillium digitatum was used originally to study precursor relationships of numerous compounds, but emphasis has now shifted to plant tissue. The elucidation of the biosynthesis of ethylene has followed the generally accepted approach: find the overall reaction in whole tissue and locate it in the cell, show how it is organized and controlled in a subcellular system, and study the details of the reaction in a purified system. Past theses from this laboratory have developed knowledge along these lines and have shown that subcellular fractions from tomatoes, beans and oats are active generators of the volatile. Mitochondria would constitute a large proportion of this subcellular particulate fraction, and the research reported in this dissertation was commenced by an investigation of ethylene evolution in relation to age controlled changes in bean cotyledon mitochondria. This was followed by the development of a technique for the preparation of a soluble enzyme system for ethylene production, from a subcellular particulate fraction of bean cotyledons. With this system, the author aspired to contribute to information on precursors for ethylene biosynthesis.

CHAPTER I

REVIEW OF LITERATURE

A. Ethylene Genesis and Biogenesis

Most of the early work concerned with ethylene biosynthesis was done on excised, senescing, diseased or otherwise injured tissues, and it was not until studies concerned with the effects of auxin on ethylene production and the ability of whole germinating seedlings to produce the gas were commenced that it became evident that ethylene is evolved by healthy, growing plants (Morgan and Hall, 1964). The theory that ethylene is a natural hormone is now generally accepted. Studies on auxin-ethylene interactions suggest that many of the effects normally attributed to auxin may actually be those of the volatile, and this has increased interest in ethylene biogenesis and the factors which control it. Early work on a biosynthetic scheme for ethylene served a definite purpose by limiting the possibilities for a pathway, but it was often very confusing, as workers using the same tissue would report conflicting results. When one appreciates the labyrinth of biochemical transformations which occur in living organisms, it is not surprising that techniques such as long term label feeding gave inconsistent results. A reconciliation was partially brought about when two possibilities were realized: (1) ethylene biosynthesis may be associated with the morphogenic stage of the tissue, and (2) more than one pathway may be operative even in the same tissue. This early work has been adequately reviewed in three recent theses (Meheriuk, 1965; Thompson, 1966; Olson, 1967).

Conventional techniques using whole tissue and even subcellular systems provided few clues regarding the natural precursors of ethylene, and thus, special techniques were instigated; one of these was the construction of model systems.

1. Model Systems

A number of model systems have been proposed and they can be classified as enzymic or nonenzymic.

(a) Enzymic

(i) Ethylene formation from methional by horseradish peroxidase

Additions of methional ($\text{CH}_3\text{-S-CH}_2\text{-CH}_2\text{-CHO}$), $\text{SO}_3^{=}$, a specific phenol, horseradish peroxidase (HRP), and Mn^{++} or H_2O_2 rapidly form ethylene (Yang, 1967). The first step in the scheme is the peroxidase-catalyzed oxidation of the phenol by H_2O_2 , and eventually, the oxidation of methional to form ethylene. Such model systems have led researchers to suggest that methional is an intermediate in the biological conversion of methionine to ethylene.

(ii) Genesis of ethylene from 'activated' linolenic acid

Lieberman and Mapson (1964) reported that a mixture of Cu^{++} , ascorbate, linolenate, and lipoxidase yielded significant amounts of ethylene. These authors postulated that the lipoxidase enzyme 'activates' the linolenate, possibly by introducing epoxide moieties. The cuprous ion, generated from Cu^{++} by ascorbate, then cleaves the 'activated' linolenate molecule to form ethylene. This system led the workers to speculate on a biological conversion of linolenate to ethylene involving lipoxidase and a copper enzyme. More recently, Lieberman and Kunishi (1967) found that propanal, a decomposition product of peroxidized linolenate, was a very efficient precursor of

ethylene in their copper-catalyzed model system, although not as efficient as methional.

(b) Nonenzymic

(i) Genesis of ethylene from a Cu^{++} , ascorbate, H_2O_2 , methionine system

Methionine in the presence of Cu^+ and an oxidizing agent was cleaved to ethylene (Lieberman et al., 1965). Warner and Leopold (1968) have reported that this conversion is greatly increased by UV irradiation or horseradish peroxidase and H_2O_2 . Methional, used in place of methionine, increased ethylene production more than sixfold, and although methional could not be identified as an intermediate, the authors were confident it represented the most direct ethylene precursor. The copper-catalyzed conversion of methional produces many volatiles whereas the enzymic system of Yang (1967) produces mainly ethylene.

(ii) Photochemical production of ethylene from methionine as mediated by flavin mononucleotide

Abeles and Rubinstein (1964) reported the first nonenzymic system for ethylene production. They discovered that the addition of flavin mononucleotide (FMN) to a concentrated dialyzate of homogenized pea seedlings resulted in evolution of the volatile. Yang, Ku, and Pratt (1967) reported that the active fraction from the dialyzate of Abeles and Rubinstein, was the amino acid methionine. A light-activated FMN removes an electron from methional and sets off a chain of reactions that eventually cleaves the postulated intermediate methional to give ethylene. As with the Cu^{++} -ascorbate system, no direct evidence was obtained implicating methional as an intermediate in the conversion of methionine to ethylene. Methional, however, increased ethylene production over methionine threefold, and there were definite similarities in ethylene production from the two sulfides.

(iii) Conversion of β -alanine to ethylene by a heat-catalyzed Snell reaction

This chemical conversion of β -alanine to ethylene (Thompson, Tribe, and Spencer, 1966) involves cupric ion and pyridoxal hydrochloride and is the least efficient of the proposed model systems.

(iv) Ethylene formation from lipid peroxides

Lipid peroxides in the presence of Cu^+ or a Cu^+ generating system (Cu^{++} and ascorbate) were found to degrade to ethylene (Lieberman and Hochstein, 1966). The degree of lipid peroxidation follows the same course as ethylene evolution, and both processes are inhibited by vitamin E. The nature of the inhibition verified the essentiality of peroxides and also suggested that the Cu^+ cleavage of the peroxidized lipid was a free radical mechanism.

(v) Ethylene formation from the ethyl moiety of ethionine

The model systems of Lieberman et al. (1965) and Yang et al. (1967) produced more ethylene from ethionine than methionine. Experiments with ethionine- ^{14}C (ethyl-1- ^{14}C) in the FMN-light model system proved to Shimokawa and Kasai (1967a) that this increase was because the ethyl moiety of ethionine, as well as carbons 3 and 4 were converted to ethylene. In addition, the ethyl group of S-ethylcysteine was also converted. These results suggested a direct natural precursor in the form of an acetaldehyde-cysteine complex.

2. Biological Systems

(a) Methionine and Methional as Ethylene Precursors

Evidence for methionine as a natural precursor of ethylene was reported by Lieberman et al. (1966). They reported a 100 percent increase in ethylene production when methionine was infiltrated into

apple tissue slices. However, this stimulation could only be achieved in fruit that had been aged beyond the natural ethylene peak. The authors suggested that other precursors may be involved in pre-climacteric evolution of the gas, whereas methionine does not become a precursor until the fruit has reached the post-climacteric stage. Gaillard et al. (1968) were unable to stimulate ethylene production with methionine in either fresh or aged discs of peel from pre-climacteric fruit, even though the discs, when aged, produced ethylene. If methionine is a precursor only in post-climacteric fruits, its importance in the overall ripening process is considerably reduced. In addition, Lieberman et al. (1966) found that methionine could not stimulate ethylene production in tissue slices of tomato and avocado. Gortner and Singleton (1965) report that methionine increases during the climacteric of the pineapple to become one of the major free amino acids.

Lieberman et al. (1966) were unable to increase ethylene production in apple tissue slices with either methional or methionine, although these compounds are very potent stimulators of the model system (Lieberman et al., 1965). Likewise, Mapson (1968) reported that methional will not stimulate ethylene evolution either in apple slices or in cauliflower florets, even though an homogenate of the latter tissue will cleave methional to ethylene. In agreement with the model system, carbons 3 and 4 of methionine formed ethylene, and both systems were inhibited by copper chelators. Burg and Clagett (1967) have also reported a stimulation of ethylene evolution by methionine in pea stem segments, previously treated with indoleacetic acid, and in apple and green banana sections. In apple sections, methionine carbons 3 and 4 were almost exclusively incorporated into ethylene. This finding agrees

with all the methionine model systems reported, as well as with Lieberman's work with apple sections. Burg and Clagett found however, that in contrast to the model systems, no volatile sulphides could be detected when L-methionine-³⁵S was incorporated into pea stem segments and apple sections. The chemical relatives of methionine, homocysteine and homoserine stimulated ethylene production from banana sections to the same degree as methionine. Moreover, the α -hydroxy analog of methionine was cleaved to ethylene in both the apple sections of Lieberman et al. (1966) and the banana sections of Burg and Clagett (1967).

The large stimulation that methional imparted to the model system of Lieberman et al. (1965) led these workers to postulate methional as an intermediate between methionine and ethylene, even though they could not stimulate ethylene evolution from apple sections with methional. Mapson and Wardale (1967) and Ku, Yang, and Pratt (1967) simultaneously reported the stimulation of ethylene formation by methional in cell free systems from cauliflower florets and pea seedlings, respectively. However, only the cauliflower extract was capable of converting methionine to ethylene. A brief note by Peters and Shorthouse (1967) reported 54% more ethylene with methional than methionine in the presence of an aqueous homogenate of Acacia georginae. Mapson and Wardale (1967) could promote ethylene evolution from intact cauliflower florets with methionine and its hydroxy analog, but not with methional. A study of floret homogenates led the authors to suggest that the particulate fraction contained the enzymes necessary for the conversion of methionine to methional, and that the soluble fraction was responsible for the cleavage of methional to ethylene.

However, the combination of both fractions gave much less ethylene than did the particulate fraction alone. Additional studies (Mapson and Wardale, 1968) have shown that two enzymes are involved in the conversion of methional to ethylene. Purification of the soluble fraction revealed an enzyme very similar to glucose oxidase and another which appeared to be a peroxidase, as predicted by Yang et al. (1967). Mapson and Wardale (1968) found that horseradish peroxidase could replace their methional cleaving enzyme, although these authors had originally maintained (Mapson and Wardale, 1967) that peroxidase had no effect on ethylene production from methional. The peroxidase enzyme cleaves methional to ethylene and requires two cofactors (supplied as a heated aqueous extract) and H_2O_2 , generated by the glucose oxidase. Mapson and Mead (1968) have identified the necessary cofactors as an ester of p-coumaric acid and methane sulphinic acid. Methionine, ethionine, and S-methylcysteine were not cleaved to ethylene by the methional cleaving enzyme.

Only minor discrepancies exist between the papers of Yang et al. (1967), Ku et al. (1967) and Mapson and Wardale (1967 and 1968), and Yang proposes that both the cauliflower and pea enzyme systems involve a peroxidase enzyme.

Some of the uncertainties in the methionine, methional scheme for the biogenesis of ethylene have been mentioned in the above treatise. A summary appears below:

- 1) Methionine has been shown to be converted to ethylene by tissue slices, but no enzymes capable of using methionine as a substrate leading to ethylene have been shown.

- 2) Methional has not been shown to occur in plants, and whether it can be formed by the action of a transaminase decarboxylase from methionine is unknown. Ku, Yang, and Pratt (1968) and Ku and Pratt (1968) have reported the enzymic formation of ethylene from the α -keto acid of methionine (α -keto- γ -methylthiobutyrate). This compound was found to rapidly convert to ethylene in the FMN model system (Yang, et al. 1967) and may well be an intermediate in the conversion of methionine to methional. Mapson (1968) reports on other compounds closely allied to methional which have been found in ripening fruit.
- 3) The ease with which methionine and methional can form ethylene both enzymically and non-enzymically in model systems should serve as a caution signal when enzymic systems are interpreted.

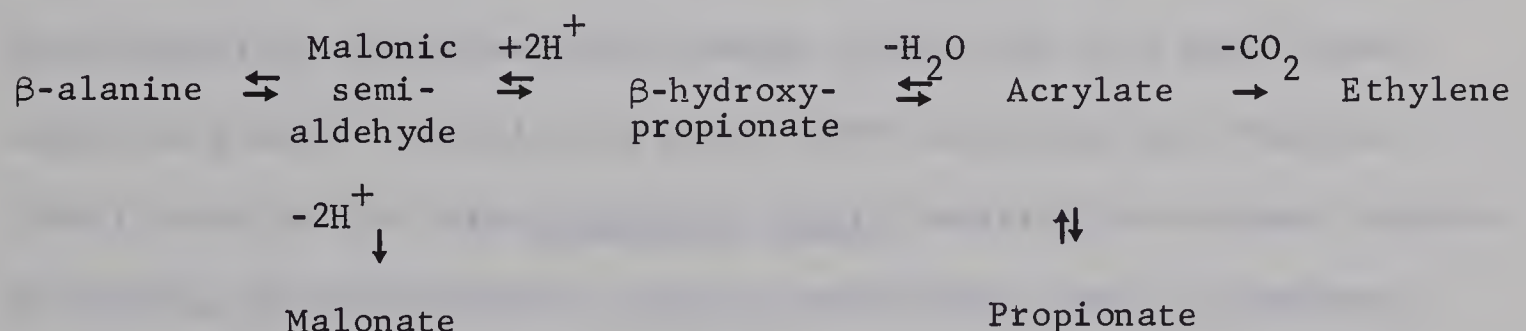
(b) Linolenate as an Ethylene Precursor

The penetration difficulties with linolenate may limit any incorporation studies involving whole or even sliced tissue (Abeles, 1966). However, Gaillard et al. (1968) carried out experiments that involved the addition of linolenate and lipoxidase to apple seed discs; no increase in ethylene evolution was observed. Lieberman and Kunishi (1967) investigated propanal, a decomposition product of peroxidized linolenate, and methionine as ethylene precursors in tomato slices; they found that propanal stimulated ethylene production 20% more than did methionine. In disagreement, Mapson (1968) could not obtain a stimulation of ethylene evolution when propanal was added to banana and apple slices. Tomato sections boosted production of the volatile only when propionic acid, not propanal, was added. Lieberman and Kunishi (1968) also reported a stimulation of ethylene evolution by propionate, but most of this stimulation resulted from a conversion of methionine carbons to ethylene.

Meigh, Jones, and Hulme (1967) studied lipoxidase activity and ethylene production in ripening fruit and found that lipoxidase activity increases with senescence and reaches a maximum just before the natural ethylene evolution peak. They suggested that this relationship could implicate lipoxidase in ethylene biosynthesis, possibly by promoting the decomposition of linolenate. Wooltorton, Jones, and Hulme (1965) showed that the majority of the ethylene came from apple peel, as opposed to apple pulp, and that apple peel contained the majority of the lipoxidase activity. Abeles (1966) expressed his doubts that linolenate could be a natural ethylene precursor when he failed to find decreases in linolenate levels following evolution of significant amounts of ethylene.

(c) β -alanine as an Ethylene Precursor

Studies on a subcellular enzyme system from bean cotyledons led Thompson and Spencer (1966) to propose the following pathway for conversion of β -alanine to ethylene.



β -alanine was found to augment by 35 percent the ethylene production from a crude powder, and the addition of certain cofactors, deemed necessary on the basis of the above proposal, increased production. Adenosine triphosphate was an essential ingredient. A reversal of the equilibrium between malonic semialdehyde (MSA) and malonate, in favour of the former, was believed responsible for the 83 percent stimulation of ethylene production when malonate was added.

Radioactive ethylene was obtained enzymically from β -alanine-2- ^{14}C , and a dilution factor of 0.57 indicated that the conversion route to ethylene was fairly direct. In addition, radioactivity was found in β -hydroxypropionate, acrylate, and malonate. Ku et al. (1967) suggest that the low conversion of β -alanine into ethylene (0.001 percent) reported by Thompson and Spencer (1967) make it apparent that β -alanine is not an immediate precursor; the failure of Burg and Clagett (1967) to obtain a stimulation of ethylene evolution when β -alanine was added to apple and banana tissue sections led them to conclude that β -alanine was not a precursor in these tissues. Wang, Jacobsen, and Tanka (1964) reported, however, that Penicillium digitatum heavily incorporated label from β -alanine-2- ^{14}C into ethylene.

The enzymes involved in the pathway for conversion of β -alanine to acrylate are well recognized in animals (Den, Robinson, and Coon, 1959; Rendina and Coon, 1957; Kupiecki and Coon, 1957) and micro-organisms (Goldfine and Stadtman, 1960), but to a much lesser extent in plants. Callely and Lloyd (1964) and Lloyd and Venables (1967) found in the alga Prototheca zopfii constitutive enzymes capable of linking the intermediates malonic semialdehyde (MSA), β -hydroxypropionate, and acrylate, of the proposed biosynthetic scheme. In no tissue is there any evidence that acrylic acid can be decarboxylated to ethylene.

An enzyme that converts β -alanine to MSA via a transaminase reaction, has been investigated in microorganisms (Roberts, Ayengar, and Posner, 1953; Goldfine and Stadtman, 1960; Hayaishi et al., 1961) and in various animal tissues (Roberts and Bregoff, 1953; Kupiecki

and Coon, 1957; Baxter and Roberts, 1958). The presence of the enzyme in plants has been little explored. Roberts and Bregoff (1953), using α -ketoglutarate (α -KG) as amino acceptor, were unable to show β -alanine transaminase activity in the pellet of an 18,000 X g centrifugation of pepper or ripening avocado homogenates. Negative results were also obtained by Kupiecki and Coon (1957) with ethanol extracts of spinach leaves when α -KG was used as an amino acceptor. The animal enzymes and most of the bacterial enzymes exhibit almost equal reactivity with either γ -aminobutyrate or β -alanine as substrate. Miettinen and Virtanen (1953) reported the presence of a strong γ -aminobutyrate transaminase in pea roots, but found no transamination between β -alanine and α -KG in this tissue. More recently, Kretovich, Karyakina, and Lyubimova (1967) reported on the presence and properties of a γ -aminobutyrate transaminase from several plant species.

Hatch and Stumpf (1962a) were able to show the presence of β -alanine- ^{14}C in several plant species after administration of propionate- ^{14}C to tissue slices, and thereby speculated on the presence of a transamination involving MSA. Tsai and Axelrod (1965) demonstrated the qualitative formation of glutamate after a 90 min incubation of β -alanine, α -ketoglutarate and a rape seedling extract.

(d) Acetaldehyde as an Ethylene Precursor

Shimokawa and Kasai (1966, 1967b) have submitted evidence to suggest that acetaldehyde may be a direct precursor of ethylene. Apple discs were able to convert more acetaldehyde- ^{14}C to ethylene, than pyruvate- ^{14}C or acetate- ^{14}C . Addition of cofactors for decarboxylation, stimulated subcellular particles from apples to convert pyruvate-3- ^{14}C , into acetaldehyde- ^{14}C . Shimokawa and Kasai proposed the formation of

an acetaldehyde-cysteine complex, which upon conversion to S-ethyl cysteine, would allow the formation of ethylene from the ethyl moiety, a reaction that took place readily in their FMN model system (Shimokawa and Kasai, 1967a).

B. Mitochondrial Aging and Ethylene Evolution

1. Mitochondrial Aging With Germination

Enzymological and ultrastructural changes in mitochondria of germinating cotyledons have received a considerable amount of attention in recent years. Cherry (1963) found that the most active and 'typical' mitochondria of peanut cotyledons appeared at 8 days; further aging resulted in decreased respiration rate, accompanied by swelling and disintegration of mitochondrial structures. Öpik (1965) reported similar results with bean cotyledons; the intact cotyledon exhibited maximum respiration 3-5 days after planting, but respiration in isolated mitochondria declined steadily after a peak at 36 h. By the third day, the cristae of mitochondria from storage cells became swollen and the matrix darkened, but these processes were delayed until the fifth day in vascular bundle mitochondria. Lado and Schwendimann (1967) found that when castor beans germinated, the mitochondria become increasingly uniform in structure before disintegration. Breidenback, Castelfranco, and Criddle (1967) demonstrated in peanut mitochondria that the increase in respiration was brought about by two superimposed phenomena: an increase in actual numbers of mitochondria, and an increase in efficiency of pre-existing mitochondria.

Akazawa and Beevers (1957) reported that the P/O values of mitochondria from germinating castor beans began at a high level and

slowly declined. Cherry (1963) measured phosphorylation of peanut cotyledon mitochondria and found it paralleled the respiration profile. Maximum P/O values for succinate and α -KG were 0.53 and 1.85, respectively. Howell (1961) and Young et al. (1960) also found that phosphorylation concurred with respiration when these researchers studied the respective mitochondria of soybean and pea cotyledons. Mitochondria from 2 day old germinating pea cotyledons did not exhibit ADP control of respiration (Young et al., 1960). Olson and Spencer (1968a) found respiratory control (RC) to be quite high in 1 day bean cotyledon mitochondria, but it had dropped slightly in 6 day old tissue.

Certain enzymes have been reported to exhibit activity vs. age profiles similar to those of other parameters. Akazawa and Beevers (1957) found that adenosine triphosphatase (ATPase) activity increased in germinating castor beans and reached a plateau at 5 days. Young et al. (1960) found that mitochondrial ATPase from germinating pea cotyledons rose rapidly after 30 h and continued to rise up to 60 h; no data was given for cotyledons aged beyond this time. Young and Varner (1959) had earlier reported that neither light nor heavy mitochondrial fractions of germinating pea cotyledons exhibited significant changes in 'phosphatase' activity (ATP as substrate) as the cotyledons aged from 2 to 7 days.

2. Association of Ethylene Evolution with Senescence

Considerable evidence has accumulated that ethylene evolution is associated with aging (Thompson, 1966). An ethylene vs. age profile was examined in whole cotyledons by Thompson and Spencer (1966), and it

was found that maximum ethylene production occurred 7 days after planting. The relationship between age and ethylene was not studied with a subcellular fraction of this tissue.

Numerous reports claim that ethylene has been produced from subcellular particulate systems from tomatoes (Chandra and Spencer, 1962; Meheriuk and Spencer, 1967), apples (Lieberman and Craft, 1961; Lieberman and Mapson, 1962), beef heart (Gibson, 1963) and rat liver (Chandra and Spencer, 1963; Lieberman and Hochstein, 1966). Ku and Pratt (1968) maintain that intact mitochondria do not produce ethylene, and that a more likely source of the volatile is a soluble enzyme outside the mitochondria. However, one should consider that the above reports on subcellular fractions do not claim that mitochondria per se produce ethylene. Indeed, Chandra and Spencer (1963) obtained ethylene from a subcellular fraction only after it had been aged in vitro. The existence of even small amounts of other cell components in the subcellular fraction could promote synthesis of the gas.

CHAPTER II

MATERIALS AND METHODS

A. Source of Chemical Materials

Routine chemicals (reagent grade) were from Fisher Scientific Co., Ltd., Eastman Organic Chemicals, or British Drug Houses, Ltd. Biochemicals were generally of the highest purity available and from the following sources: L-methionine- ^{14}C (U) (>99%), β -alanine-2- ^{14}C (>99%), malonate-2- ^{14}C (>98%), PPO (2,5-diphenyloxazole), and POPOP (p-bis2-(5-phenyloxazoloyl)-benzene) from New England Nuclear; tritiated water (99%) from Volk Radiochemicals; ethylene- ^{14}C (U) (>98%) from Nuclear Chicago Corporation; ATP, thiamine pyrophosphate, α -KG, HEPES, ADP, dithiothreitol, and BSA (fraction V, B grade) from Calbiochem; L-methionine from Mann Research Chemicals; Tricine from General Biochemicals; NADPH, NADH, cytochrome c (type III from horse heart), OAA, pyruvate, human serum albumin (Grade III), and rotenone from Sigma Chemical Company; methional and sodium acrylate from K and K Co., Sunset Blvd., Hollywood, Calif.; Triton X-100 (octylphenoxypolyethoxyethanol) from Applied Science Laboratories; ethylene (USP 99%) from Ohio Chemical and Manufacturing Company; sulfonic acid cation exchange resin (AG 50W X 12, 50-100 mesh) from Biorad Laboratories; glutamate-pyruvate and glutamate-oxalacetate transaminase kits from Boehringer Mannheim Corp., Market St., San Francisco; Sephadex G-25 from either Pharmacia or Sigma Chemical Company; pyridoxal phosphate from either General Biochemicals or Nutritional Biochemicals; TES was either synthesized as outlined in the appendix or purchased from General Biochemicals and recrystallized

from ethanol; silica gel G from Canadian Laboratory Supplies; Tween 80 (polyoxyethylenesorbitanmonooleate) from Atlas Chemical Corporation; Cab-O-Sil (#M-5) from Cabot Corporation, Boston. All water used was double distilled deionized and contained < 2 ppm ionizable impurities.

B. Methods Used to Study Mitochondrial Activity With Germination

1. Growth of Seedlings

Seeds of Phaseolus vulgaris L. var. Kinghorn wax were surface sterilized by soaking for 1 min in a 10% solution of household bleach (5.25% available chlorine), thoroughly washed, planted in horticultural grade vermiculite, and grown at 26.0 C in the dark. The cotyledons were picked on specific days; day 1 was 24 h after planting, etc.

2. Preparation of Mitochondria

Cotyledons were gently crushed (3 ml medium per g of cotyledon) for 1.5 min with a mortar and pestle in the following 'grind buffer': 0.5 M mannitol, 0.001 M EDTA, 1% BSA (w/v), and 5 mM TES, pH 7.4 (NaOH) at 0 C. The brei was filtered through cheesecloth and the resulting filtrate subjected to differential centrifugation in an International Model B20 refrigerated centrifuge. The supernatant from the first spin at 2,500 X g for 10 min was centrifuged at 10,000 X g for 15 min. The pellet obtained was resuspended in the following 'wash medium' (5 ml/17 g cotyledons): 0.3 M mannitol, 0.001 M EDTA, 0.1% BSA (w/v), and 5 mM TES, pH 7.2 (NaOH) at 0 C. This suspension was divided into two portions, one containing twice as much suspension as the other. The supernatant from a centrifugation at 2,500 X g for 10 min was subsequently centrifuged at 10,000 X g for 15 min. The larger pellet (used for RC, ADP/O values, and respiration studies) was surface washed

with 'assay medium' of the following composition: 0.3 M mannitol, 4 mM MgCl_2 , 2.5 mM KH_2PO_4 , and 1 mM TES, pH 7.2 (NaOH) at 24 C. The pellet was suspended in approximately 0.3 ml of this buffer by gently swirling with a vortex mixer. The smaller pellet (used for ATPase assay) was surface washed with 'ATPase buffer' of the following composition: 0.3 M mannitol, 4 mM MgCl_2 , and 1 mM TES, pH 7.2 (NaOH) at 24 C. The pellet was suspended in a small amount of this buffer.

3. Respiratory Parameters

(a) Respiration Rates

Respiration rates were measured concomitant with respiratory control (RC) and ADP/O values. Approximately 0.15 ml of mitochondrial suspension was added to 3.0 ml assay medium, and the time for complete exhaustion of oxygen was determined after 2 additions (0.150 μmoles each) of ADP. Oxygen uptake was measured at 24.0 C with a Model 53 oxygen monitor and a Clark fixed-voltage polarographic probe (Clark, 1956) (Yellow Springs Instrument Company, Yellow Springs, N.J.). Respiration rate with no added substrate was subtracted from the rate with substrate. A Beckman 100-mV potentiometric recorder (chart speed 0.5 in/min) provided a continuous tracing of oxygen concentration with time.

The Lowry method (Lowry et al., 1951) for the quantitative determination of protein was modified to determine mitochondrial protein concentration. To remove compounds that interfere with the protein assay, the mitochondria were first precipitated by addition of 50 μl 40% trichloroacetic acid (TCA) (w/v) to 0.3 ml suspension, followed by centrifugation at 1200 X g for 5 min. The pellet was

washed twice with 1 ml 5% (w/v) TCA and then dissolved in 0.5 ml of 1 N NaOH in a boiling water bath for 5 min. Subsequent steps included: making to a volume of 1 ml with water, addition of 5.0 ml CuSO_4 -potassium tartrate reagent, and addition of 0.5 ml of 1.0 N phenol reagent as described by Lowry et al. (1951). After 30 min the absorbance was determined with a Beckman DU2 spectrophotometer at 750 m μ . Human serum albumin in water was used as a standard and treated similarly. Assays were carried out in triplicate. The solubility of oxygen in the assay medium was determined by the Winkler (1914) method and was found to be 225 μM .

(b) Respiratory Control and ADP/O Values

RC values were calculated in accordance with the definitions of Chance and Williams (1955, 1956), that is, the ratio of respiration rates in state 3 (phosphate, oxygen, substrate, and ADP in excess) and state 4 (ADP limiting). Two additions of ADP, each of 0.150 μmoles , were made to 3.0 ml of assay medium containing mitochondria and either 17 mM succinate, 17 mM α -KG and 8 mM malonate, 17 mM malonate, or 34 mM propionate. ADP/O ratios were determined from the amount of oxygen utilized during state 3, responding to 0.150 μmoles of ADP.

(c) ATPase Activity

Mitochondrial hydrolysis of ATP was assayed by inorganic phosphate release with time, according to the method of Mozersky, Pettinati, and Kolman (1966). Each sample cell contained 3 ml ATPase buffer (see mitochondria preparation B.2), 2 μmoles ATP and 0.1 ml mitochondrial suspension at 24 C. The contents of each cell were kept well mixed by a magnetic stirrer. Controls, lacking either ATP or mitochondria, were run on alternate days. After addition of the

mitochondria, 0.2 ml of the sample were removed immediately and pipetted into 5.8 ml of protein precipitant (1.2 M NaClO_4 , 80 mM glycine, and 0.2 M HCl, final concentration). Subsequently, samples were taken at 1 minute intervals up to 9 minutes. The protein was removed by centrifugation, and 4 ml of the supernatant were treated as outlined by Mozersky et al. (1966). Five μg phosphate per 0.2 ml of sample to be assayed (the upper limit of the assay) gave an absorbance of 0.650. The Pi present at each sampling was plotted against time; the slope was determined to give a rate of hydrolysis. Duplicate values were averaged. Protein was determined as outlined under respiration rates (B.3.).

4. Electron Microscopy

Mitochondria were prepared as described under respiration rates with two exceptions; the brei was centrifuged at 5,000 X $\underline{\text{g}}$ rather than 2,500 X $\underline{\text{g}}$, and the final centrifugation was omitted. Glutaraldehyde fixative (10 ml containing 50 mM TES, 6% glutaraldehyde and 0.3 M mannitol, pH 7.3 (NaOH) at 0 C) was mixed with 10 ml mitochondrial suspension and the mixture allowed to stand in ice for 3 h. After centrifugation at 10,000 X $\underline{\text{g}}$ for 15 min, the pellet was washed (very gently) in 0.05 M phosphate buffer (pH 7.3 (NaOH) at 0 C) containing 0.3 M mannitol. After several washings, the pellet was added to 10 ml osmium tetroxide (OsO_4) solution (1% OsO_4 in 0.05 M phosphate buffer) and allowed to stand in ice for 2 h. The pellet was dehydrated in an ethanol series, and 10 ml propylene oxide were added. The propylene oxide was renewed after 10 min, and after an additional 10 min, the pellet was transferred to an epon:propylene oxide (1:1) mixture overnight, then transferred to pure epon and hardened at 60 C. Silver sections (approximately 600-900 Å) were cut on an LKB microtome and

stained for 3 min with uranyl acetate (Stempak and Ward, 1964) and 4 min with lead citrate (Reynolds, 1963) on formvar coated copper grids. The grids were examined in a Phillips 100 electron microscope at 60 KV.

C. Ethylene Production from a Subcellular Particulate Fraction of Germinating Beans

1. Preparation of Subcellular Fraction

Seedlings were grown as outlined above (B.1.). A subcellular particulate fraction was prepared by grinding 70 g cotyledons with a conventional meat grinder in the following 'grind buffer' (2 ml buffer/g cotyledon): 0.3 M mannitol, 5 mM TES, pH 7.4 (NaOH) at 0 C. Cheesecloth filtration was followed by centrifugation at 2,500 X g for 10 min. The supernatant was centrifuged at 25,000 X g for 20 min to produce a pellet that was suspended in 20 ml water containing 1.5 mM ATP. Ethylene collection was at pH 7.0 for 0-2 and 2-4 h.

2. Analysis of Ethylene Evolved

Ethylene was collected by passing purified air over the gently stirred sample, through a CO₂ adsorbent (lithasorb, Fisher Scientific Co.) and 'drierite'. In most cases the sample volume was 20 ml and all collections were at room temperature. A full cofactor complement is given under D.1. below. Ethylene was adsorbed on 0.5 g silica gel (Davison, grade 15, 35-60 mesh) in a glass tube at dry ice temperatures. The tube was maintained at this temperature and connected to a two-way valve inserted into the helium input line of a Perkin Elmer Model 811 flame ionization gas chromatograph. With the valve closed, the tube was heated to 40 C, thereby releasing the ethylene. The two-way valve was then opened and the contents of the tube flushed onto a 50 cm X 6 mm ID column that contained activated alumina with 2½% silicone 550. Column temperature was 20 C and detector temperature was 125 C. A standard

curve was made by injecting into the gas chromatograph, with a gas tight syringe, known amounts of ethylene. Peak height vs. μ l ethylene was plotted.

D. Conversion of β -alanine to Ethylene

1. Preparation of the Subcellular Particulate Enzyme System

(a) Isolation of a Subcellular Particulate Fraction

Bean seedlings were grown as outline above (B.1.) with the exception that the seeds were not surface sterilized. Cotyledons, $3\frac{1}{2}$ days old, were ground as in C.1. above. The supernatant layer, obtained after cheesecloth filtration and centrifugation at $2,500 \times g$ for 10 min, was centrifuged at $32,000 \times g$ for 15 min. The pellet recovered was resuspended in 1 mM TES, pH 7.6 at 0 C (1 ml/8 g cotyledon) and freeze-dried.

(b) Cofactor Stimulation of Ethylene Production from a Lyophilized Particulate Fraction

Two collections were set up, each containing 0.5 g lyophilized particulate fraction in suspension and 20 mM TES, but only one contained the cofactors necessary for the conversion of β -alanine to ethylene as proposed by Thompson and Spencer (1966) (50 mM α -KG, 1.5 mM ATP, 0.17 mM CoA, 2.0 mM TPP, 1.0 mM $MgSO_4$, and 0.5 mM pyridoxal phosphate).

(c) 'Solubilization' of the Particulate Fraction

(i) Sonication

0.5 g lyophilized particulate fraction was suspended in 10 ml buffer (4 mM TES, pH 7.6 at 0 C) and sonicated for 4 min at 1.0 amp with the temperature maintained below 7 C. The suspension was centrifuged at $100,000 \times g$ for 15 min and lyophilized to remove endogenous ethylene that had formed by sonication. The dry powder was dissolved

in 20 ml H_2O containing 50 mM β -alanine, 50 mM malonate, 50 mM α -KG, 1.5 mM ATP, 0.17 mM CoA, 2.0 mM TPP, 1.0 mM $MgSO_4$, and 0.5 mM pyridoxal phosphate, pH 7.0, and ethylene collected for 2 h by the method outlined in C.2.

(ii) Osmotic Rupture

0.5 g lyophilized particulate fraction was suspended in 10 ml buffer (4 mM TES, pH 7.6 at 0 C) and allowed to stir gently in ice for 1 h. The suspension was centrifuged at 100,000 X g for 15 min, 10 ml water containing the cofactors of (i) were added to the supernatant, and the ethylene evolved over a 2 h period was collected.

(iii) Osmotic Rupture + 1% Tween 80

Treatment and collection were exactly as in (ii) with the exception that solubilization was in the presence of 1% Tween 80.

(iv) Osmotic Rupture + 1% Triton X-100

Treatment and collection as outlined in (ii) and (iii), but 1% Triton X-100 replaced 1% Tween 80.

(v) Osmotic Rupture + 0.4% Triton X-100

0.4% Triton X-100 replaced the more concentrated detergent outlined in (iv), and the procedure was as before.

Further investigation of the treatment led to the routine use of the following technique. Two g of the freeze-dried subcellular fraction were treated with 40 ml of Triton buffer (0.4% Triton X-100 and 10 mM TES, pH 7.6 (NaOH) at 0 C) for 1 h to initiate solubilization. Centrifugation at 100,000 X g for 30 min yielded a supernatant that was chromatographed on Sephadex G-25, with 0.1% Triton X-100 and 10 mM TES solution (pH 7.6 at 0 C) as the eluting buffer. Approximately 1 mg of pyridoxal phosphate was added routinely to check column separation.

Gel filtration (to remove small molecules from the protein) was carried out on a 36 cm x 3.2 cm column maintained at 4 C. The protein was monitored visually. The pellet was resuspended in 35 ml Triton buffer and allowed to stir gently overnight at 0 C. Subsequent centrifugation and chromatography were as above. The two effluents were combined; approximately 80 ml of enzyme solution were obtained for the ethylene collections. Early experiments were at pH 7.0, but in order to protect NADH from decomposition at acid pH, later collections were at pH 7.2. All collections were at room temperature.

2. Inhibition of Ethylene Biosynthesis by Heating, Arsenite or Urea

The various treatments were carried out as follows:

- 1) Control. Fifteen ml of enzyme solution were freeze-dried.
- 2) Heating. Fifteen ml of enzyme solution were heated to 95 C and were maintained there for 15 min. The solution was then freeze-dried.
- 3) Urea. Sufficient urea was added to obtain a concentration of 8 M. The sample pH was adjusted to 7.0 and the sample freeze-dried.
- 4) Arsenite. Sodium arsenite (0.1 M) was added to 15 ml of enzyme solution. The solution was adjusted to pH 7.0 and freeze-dried.

3. Isolation of a Cytoplasmic Enzyme System

The supernatant that contained the cytoplasmic enzymes, obtained from the isolation of the particulate fraction (isolated as described in 1.(a)) was brought to pH 7.6 at 0 C and centrifuged at 100,000 X g for 10 min. The supernatant was freeze-dried and 80 ml of buffer (10 mM TES and 0.1% Triton X-100, pH 7.6 at 0 C) were added. Undissolved material was removed by centrifugation at 100,000 X g for

10 min. The supernatant (40 ml) was chromatographed on Sephadex G-25 as outlined in (c)(v) above.

4. Determination of Radioactive Ethylene

Ethylene was collected as outlined in C.2. above. A flow splitter was utilized to direct 80% of the sample to a Nuclear Chicago gas radiochromatograph (GRC) and the remaining 20% to the gas chromatograph flame. The GRC (85 ml chamber flushed with high purity methane at 30 cc/min) when connected to a 10 mv potentiometric recorder provided a record of radioactivity in the column effluent. The instrument was standardized, by the injection of known amounts of ethylene- ^{14}C (U) into the GC and plotting peak height vs. dpm of ethylene.

5. General Studies

These experiments were conducted on the enzyme system described above. They involved an investigation of levels of β -alanine, malonate, NADH, NADPH and protein, and the effect of pH, inhibitors, cofactors, α -KG, intermediates, and DTT, on ethylene production.

6. Possible Steps in the Conversion of β -alanine to Ethylene

(a) Malonic Semialdehyde- ^{14}C from β -alanine-2- ^{14}C

(i) Formation of 2,4-dinitrophenyl hydrazone of malonic semialdehyde

The reaction mixture contained 1 μC (2.2×10^6 dpm), β -alanine-2- ^{14}C ($3.43 \mu\text{C}/\mu\text{mole}$), cofactors and approximately 82 mg protein. After a 2 h incubation, 4 ml were removed and 0.57 ml 40% TCA added (final TCA concentration 5%). The precipitated protein was removed by centrifugation at $26,000 \times g$ for 5 minutes. A small

amount of cold malonic semialdehyde (MSA) (see subsequent section for synthesis) was added and the 2,4-dinitrophenyl hydrazones made by the addition of sufficient 2,4-dinitrophenyl hydrazine (recrystallized from ethanol) solution (0.2% (w/v) in 2 N HCl).

(ii) Thin-layer chromatography and radioactivity of MSA-2,4-dinitrophenyl hydrazone

Extraction with three 20 ml portions of CHCl_3 :ethanol (4:1), evaporation to dryness under N_2 , and addition of 1.0 ml of solvent provided a sample suitable for TLC spotting. TLC was carried out on glass plates coated with 0.5 mm of silica gel G (30 g silica gel G and 60 ml water coated four 20x20 cm plates). Plates were air dried after coating and activated for 1 h just before use at 105 C under 'house vacuum'. The plates were streak spotted (approximately 10 μl of sample/cm) and developed in one or more of the following solvent systems: (a) benzene:acetic acid (19:1), (b) triethylamine:ether:water:pyridine (60:40:20:20), (c) ethanol:butanol:0.5 M ammonia (70:50:15). The yellow 'hydrazone' of MSA was scraped off the plate and either extracted with solvent and respotted, or added to a 1,4-dioxane-Cab-O-Sil fluor (7.0 g PPO (2,5-diphenyloxazole), 0.3 g POPOP (p-bis2-(5-phenyloxazoloyl)-benzene), 100 g naphthalene, and 1 l 1,4-dioxane) and the radioactivity determined with a Nuclear Chicago Unilux II liquid scintillation system.

To determine the effects that silica gel and hydrazone had on the counting procedure, a quench curve was made. Various amounts of cold hydrazone were spotted on silica gel TLC plates and the areas cut out and placed in scintillation vials. Amounts of hydrazone solution spotted ranged from 0-100 μl . β -alanine-2- ^{14}C (110,000 dpm) were

added to each vial, the counting efficiency determined and a quench curve constructed (Unilux II operators manual, 1967).

(b) Stimulation of Ethylene Biosynthesis by Addition of Postulated Intermediates

(i) Synthesis of malonic semialdehyde
is

Malonic semialdehyde^Δdecarboxylated to acetaldehyde quite readily (50% in 24 h) (Robinson and Coon, 1963) and therefore must be made just prior to use. However, the diacetal ester of MSA is quite stable as is the alkaline solution of β,β -diethoxypropionate, a compound that is readily formed by the addition of alkali to the diacetal ester. β,β -diethoxypropionate is cleaved by HCl to form MSA. The synthesis of the diacetal ester was carried out as described by Robinson and Coon (1963). Ketene, generated by the method of Williams and Hurd (1940), was bubbled through a suspension containing triethylorthoformate (redistilled). The diacetal ester (ethyl β,β -diethoxypropionate) formed was purified by distillation at 1.5 mm (bp 54 C). Conversion to β,β -diethoxypropionate was accomplished by the addition of 0.5 N NaOH (15 ml/g diacetal ester) with vigorous shaking. The β,β -diethoxypropionate solution was kept at -20 C, and readily gave MSA when thawed and an equal volume of 4.5 N HCl added. A yield of 80% from the diacetal ester was assumed.

(ii) Synthesis of β -hydroxypropionate

This compound is unavailable commercially in a pure form, and therefore was synthesized according to the method of Robinson and Coon (1963). β -hydroxypropionic acid, generated from propiolactone, is purified as the crystalline calcium-zinc salt. Solutions of potassium β -hydroxypropionate were then generated as needed by passing

a solution of the calcium-zinc salt through a column of Bio-rad AG 50 W x 12, 50-100 mesh in the K^+ form.

(c) Incorporation of Tritium into Ethylene

Enzyme solution was prepared as described under D.1. above, and a portion corresponding to 100 mg of protein was freeze-dried. The powder was dissolved in TES buffer that contained the normal cofactors, β -alanine, and malonate. Two experiments were conducted; in the first (final volume 10 ml) the specific activity of tritiated water was $0.270 \mu\text{c}/\mu\text{mole}$, whereas in the second (final volume 8 ml), it was $0.338 \mu\text{c}/\mu\text{mole}$.

(d) Presence of a Transaminase Converting β -alanine to Malonic Semialdehyde

(i) Malonic semialdehyde standard curve

Malonic semialdehyde (MSA) was prepared as described above. The MSA solution was brought to pH 5.2 (NaOH) and an appropriate dilution made. Aliquots were pipetted out in triplicate and brought to a volume of 1 ml with water; 1 ml of water served as a blank. Each sample received 0.2 ml 2 M sodium acetate, pH 5.2; subsequent steps were as outlined under Transaminase assay.

(ii) Transaminase assay

The enzyme prepared as described in D.1. above, was assayed according to the method of Hayaishi and Nishizuka (1962). The standard assay at pH 7.2 contained $60 \mu\text{moles}$ of both β -alanine and amino acceptor, $150 \mu\text{moles}$ TES, and approximately 2.0 mg of protein in a final volume of 3.0 ml. Incubation was at 33 C in a shaker bath, and the reaction was stopped by the addition of 0.43 ml of 40% TCA (final concentration of TCA, 5%). Protein was removed by centrifugation at $26,000 \times g$ for 5 minutes. Two, 1 ml aliquots of the supernatant were

brought to pH 5.2 by the addition of 0.2 ml of 2 M sodium acetate in 0.91 N NaOH. The amount of MSA formed was quantitatively determined by formation of the formazan as outlined by Hayaishi and Nishizuka (1962). The formazan was extracted from the water by the addition of 4 ml ethyl acetate. The absorbance of the organic layer was measured at 440 m μ in a Beckman DU2 spectrophotometer; samples were normally blanked against zero time.

(iii) Properties of the enzyme

The reactivity of the enzyme with pyruvate, OAA, and α -KG as amino acceptors was investigated. In addition, the necessity of pyridoxal phosphate addition was examined by the incubation of the enzyme with pyridoxal phosphate, removal of the unbound cofactor by Sephadex G-25 gel filtration (D.1. above), and subsequent addition of pyridoxal phosphate back to the enzyme. The enzyme activity of this sample was compared to a control that had no additional cofactor. A system that contained mainly cytoplasmic enzymes was prepared as outlined in D.3. above, and the activity of the cytoplasmic transaminase, with pyruvate and α -KG as amino acceptors, was examined.

(iv) Identification of malonic semialdehyde as the reaction product of the transaminase

MSA was identified as the 2,4-dinitrophenyl hydrazone. The hydrazone was made and chromatographed as outlined in 6.(a) above; the chromatography solvent used was benzene:acetic acid (19:1). The area on the plate corresponding to MSA was scraped off and the hydrazone extracted. The sample was heated for 1 h in a boiling water bath to decarboxylate the MSA-hydrazone and form the acetaldehyde-hydrazone. The sample was rechromatographed, and the plate was examined for the 2,4-dinitrophenyl hydrazone of acetaldehyde.

E. Conversion of Methionine to Ethylene

1. By Excised Leaves

Seedlings of Phaseolus vulgaris were planted in California soil mix and grown in a 14 h light day at 65 F. The primary leaves were removed from the plant by cutting under water at an age (16-19 days) when they were evolving considerable amounts of ethylene. Three leaves (fresh weight approximately 1 g/leaf) were transferred to a small beaker containing the radioactive amino acid and were allowed to take up L-methionine- ^{14}C (U) under moderate light (approximately 150 ft c). They were placed in a large chamber, and the ethylene evolved was collected. Radioactive ethylene was determined as in D.4. above.

2. By a Solubilized Particulate Fraction

(a) General Studies

Experiments to examine the effects of methionine and methional on ethylene production were carried out on the soluble system described above (D.1.). The necessity of cofactors in the system with these sulfides, and the performance of a heat-treated enzyme were considered.

(b) Radioactive Studies

Two experiments were conducted in an attempt to note the production of ethylene- ^{14}C from L-methionine- ^{14}C (U); one collection contained 5 μc L-methionine- ^{14}C (U) (17.15 $\mu\text{c}/\mu\text{mole}$), and protein and cofactors as usual. Ethylene was collected for 0-2 and 2-4 h, and radioactivity was detected as outlined previously (D.4.). A second collection varied from the first in that 10 μc L-methionine- ^{14}C (U) (0.4 $\mu\text{c}/\mu\text{mole}$) were added, and ethylene collection was for 0-8 and 8-18 h.

(c) Methional as an Intermediate

Collections that contained cofactors were set up as usual, but to identify methional, 5 μ c of L-methionine- ^{14}C (U) were added. Four ml aliquots were taken after 2 and 4 h of collection and treated as outlined for MSA under D.6. above. Identification of the 2,4-dinitrophenyl hydrazone of methional- ^{14}C was made by obtaining a significant and constant number of dpm when the sample was chromatographed in one or more of the following solvent systems:

(a) benzene:acetic acid (19:1), (b) water saturated benzene, (c) benzene:petroleum ether (bp 30-60 C) (3:1). The heat lability of the system that converts methionine to methional was checked by heating the enzyme solution for 10 min in a boiling water bath before the addition of L-methionine- ^{14}C (U).

F. Conversion of Miscellaneous Compounds to Ethylene

Propionic acid or propanal were added to collections that contained enzyme solution and cofactors, and the effect of these compounds on ethylene production was noted. When results indicated possible non-enzymic production of the volatile, experiments with heat-denatured protein were executed.

CHAPTER III

MITOCHONDRIAL AGING AND ETHYLENE EVOLUTION

A subcellular particulate fraction from bean cotyledons was shown to evolve ethylene, and a study of the amount of ethylene evolved by this fraction as germination proceeded, indicated a definite pattern. Since mitochondria made up the majority of this subcellular fraction, it was felt that an enzymic as well as a structural examination of the mitochondria might yield information concerned with ethylene biosynthesis.

A. Mitochondrial Activity with Germination

1. Respiratory Parameters

(a) Respiration Rates

Respiration rates of isolated mitochondria with succinate or α -ketoglutarate (α -KG) as substrate are shown in Figure 1. Only one determination per day is given. A second complete experiment showed identical trends. In agreement with Howell (1961, soybean cotyledon mitochondria) and Cherry (1963, peanut cotyledon mitochondria), succinate was a much more efficient substrate than α -KG (succinate respiration was three times α -KG respiration at 7 days). Öpik (1965, wax bean cotyledon mitochondria) found that succinate respiration was only 20% higher than α -KG respiration in mitochondria from 1 day cotyledons, and that 3 day cotyledons would not oxidize α -KG. In the present experiments, malonate (8 mM) was always used with α -KG to inhibit succinate supported respiration. The succinate supported respiration in mitochondria from 5 day old cotyledons was 97% inhibited by 8 mM malonate. It should be noted that malonate could significantly contribute to α -KG metabolism, especially in older tissue (Figure 2).

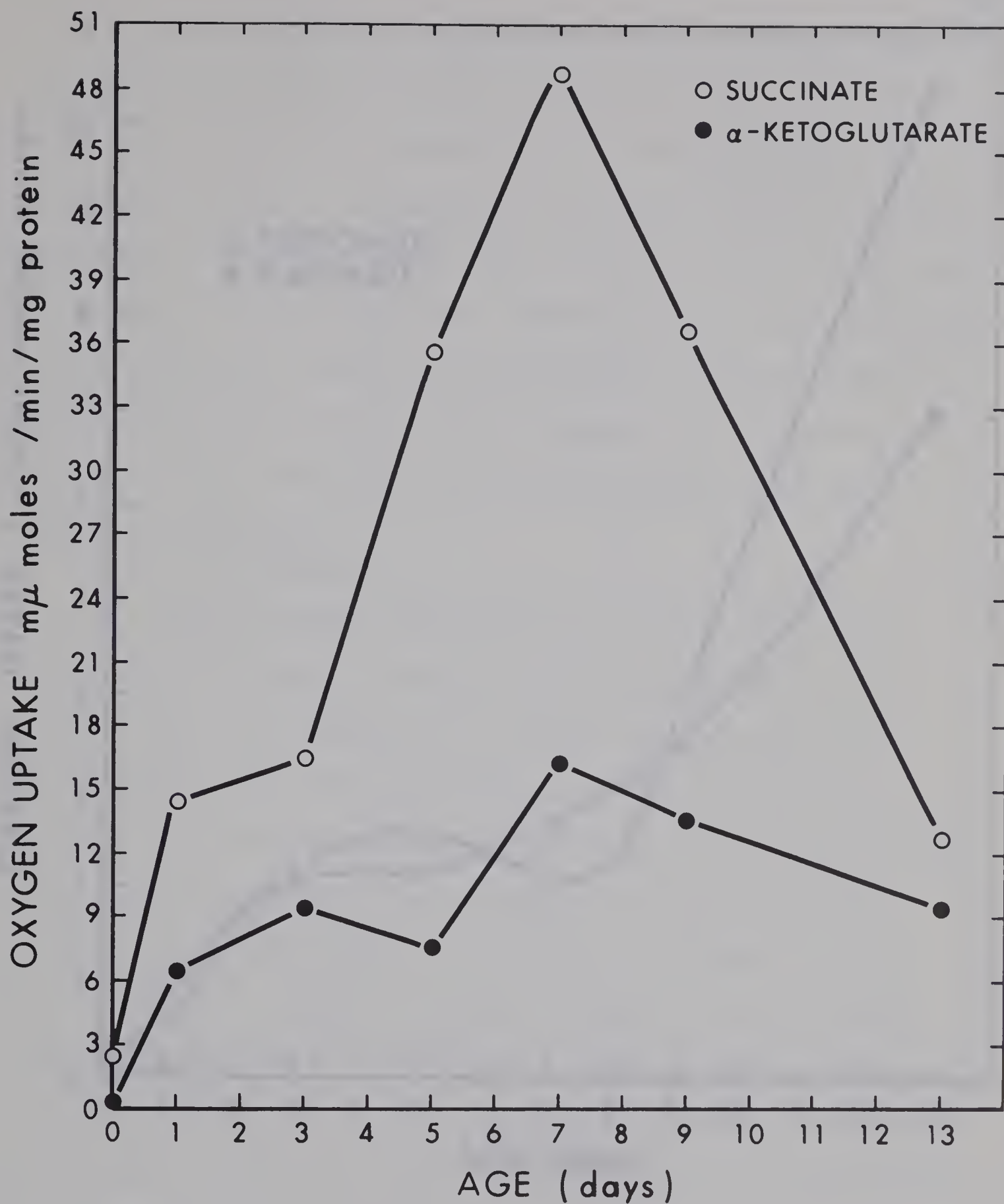


Figure 1. Respiration rates, with succinate or α -ketoglutarate as substrate, of mitochondria isolated from cotyledons of germinating bean seedlings. Rates were measured concomitant with RC and ADP/O ratios. Assay medium: 17 mM succinate or 17 mM α -KG and 8 mM malonate, 4 mM MgCl_2 , 2.5 mM KH_2PO_4 , and 1 mM TES, pH 7.2. Final volume was 3.2 ml and assay temperature 24 C.

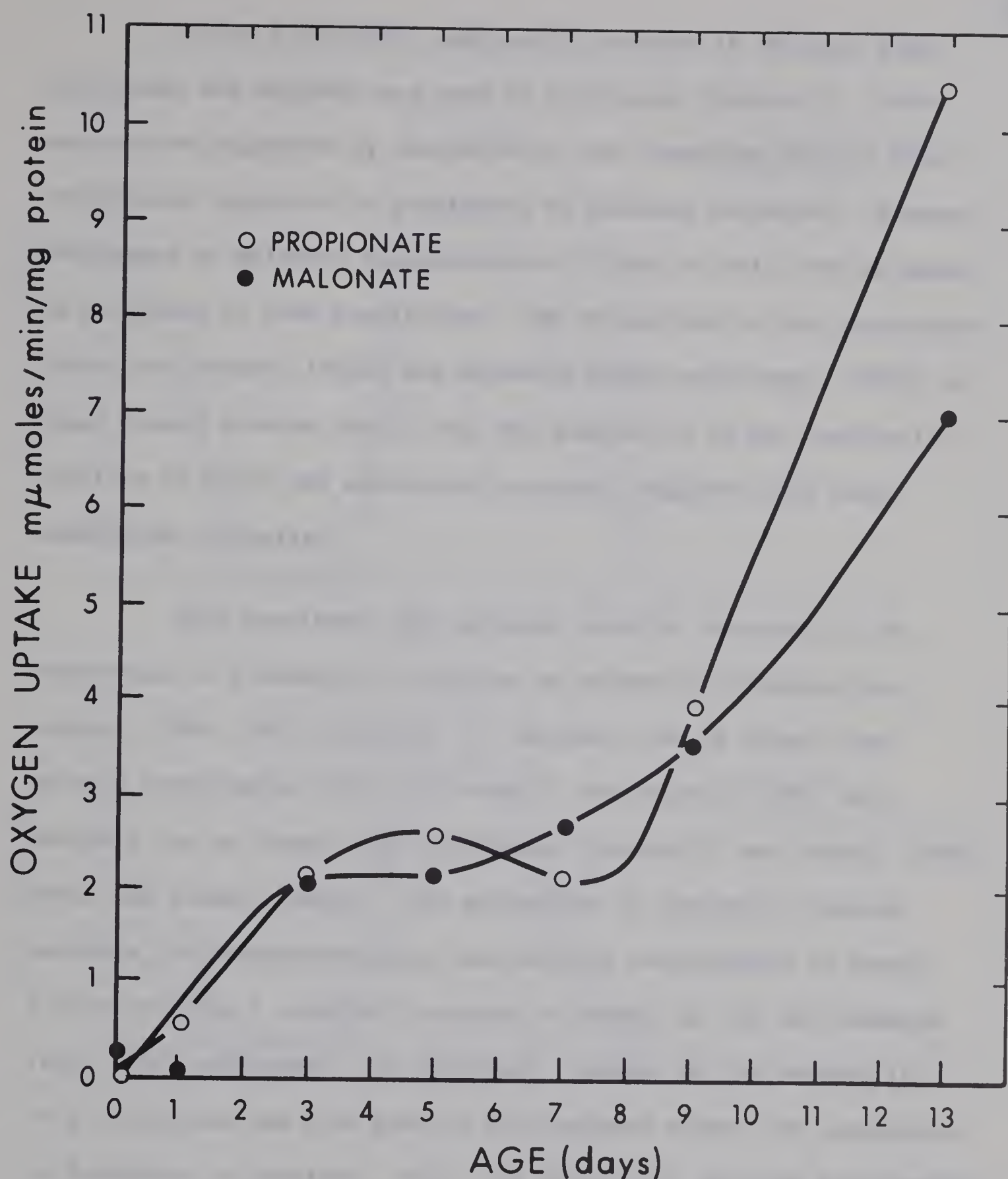


Figure 2. Respiration rates, with malonate or propionate as substrate, of mitochondria isolated from cotyledons of germinating bean seedlings. Assay medium: 17 mM malonate or 34 mM propionate, 0.3 M mannitol, 4 mM MgCl_2 , 2.5 mM KH_2PO_4 , and 1 mM TES, pH 7.2. Final volume was 3.2 ml and assay temperature 24 C. No RC was obtained with either of these two substrates.

Quite a different respiration pattern is obtained when propionate and malonate are used as substrates (Figure 2). While respiration supported by succinate or α -KG decreases after 7 days, respiration supported by propionate or malonate increases. However, propionate or malonate respiration at 13 days is still not as great as succinate or α -KG respiration. The metabolism of both propionate (Hatch and Stumpf, 1962a) and malonate (Hatch and Stumpf, 1962b) in plant tissue produce acetyl CoA; the similarity in the respiration profiles of these two substrates certainly suggests that their metabolism is similar.

Both propionate and malonate could be involved in the conversion of β -alanine to ethylene as proposed by Thompson and Spencer (1966, 1967) (Chapter I). Malonate can be formed from malonic semialdehyde (MSA) (Giovanelli and Stumpf, 1958), and acrylate can be formed from propionate (Giovanelli and Stumpf, 1958; Hatch and Stumpf, 1962a). The metabolism of propionate through acrylate, β -hydroxypropionate, and malonic semialdehyde to acetyl CoA constitutes a modified β -oxidation scheme for the odd numbered fatty acid, propionate. As mentioned, several of the enzymes in this conversion are also part of the proposed scheme for conversion of β -alanine to ethylene. Thus, the ability of isolated mitochondria to metabolize propionate indicates that at least a portion of the scheme for conversion of β -alanine to ethylene is operative in these cotyledons.

(b) Respiratory Control and ADP/O Values

These two parameters displayed marked changes during germination (Figure 3). At day 1 the mitochondria exhibited very

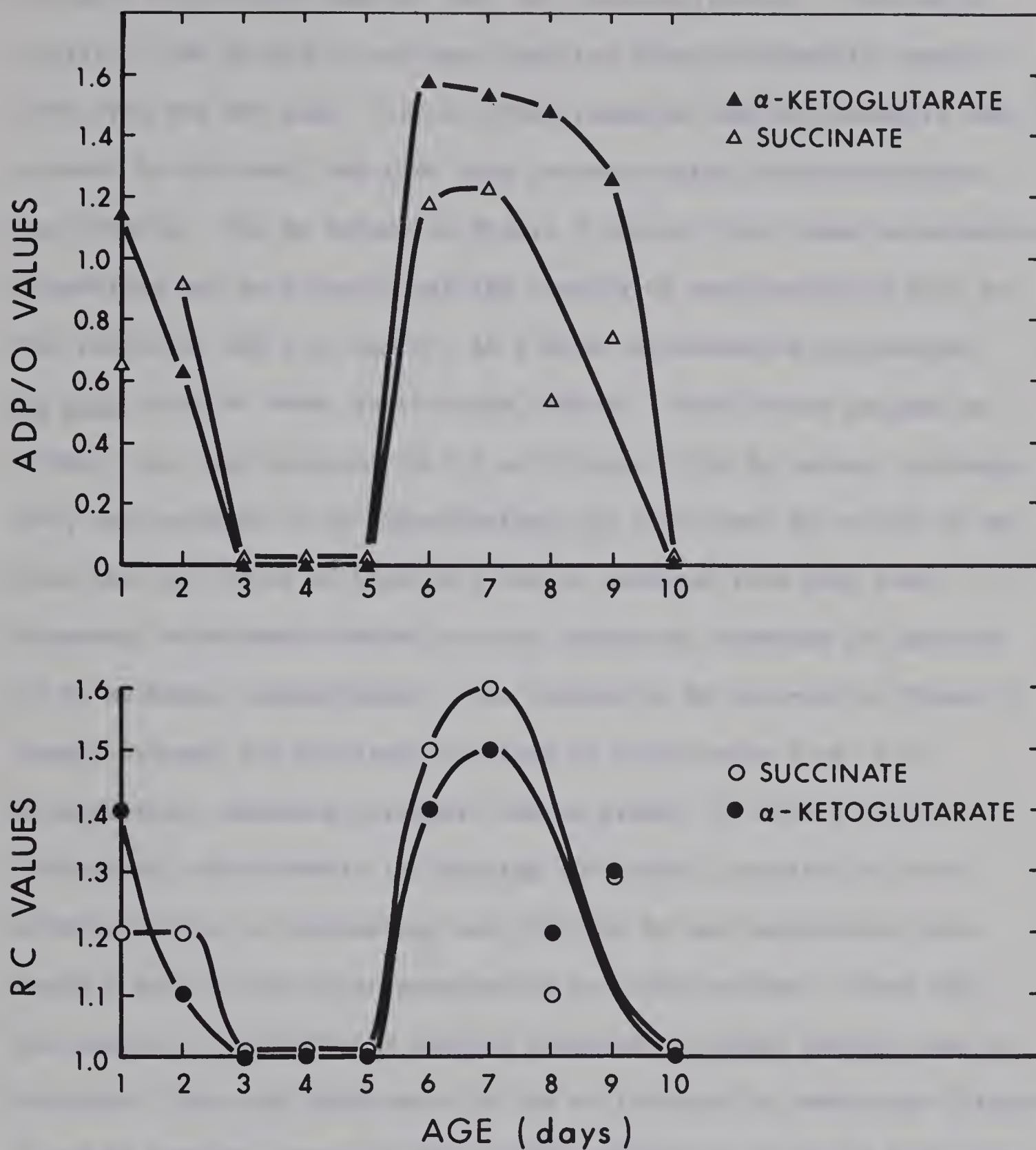


Figure 3. Respiratory control and ADP/O values, with succinate or α -ketoglutarate as substrate, of mitochondria isolated from cotyledons of germinating bean seedlings. Assay medium: 17 mM succinate or 17 mM α -KG and 8 mM malonate, 4 mM MgCl_2 , 2.5 mM KH_2PO_4 , 0.3 M mannitol, and 1 mM TES, pH 7.2. Final volume was 3.2 ml and assay temperature 24 C.

definite respiratory control (RC) and phosphorylation. This early control, lost by day 3, may have resulted from mitochondria carried over from the dry seed. Yatsu (1965) reported that mitochondria are present in dry seed, and that they possess typical double membranes and cristae. The RC values of Figure 3 suggest that these mitochondria degenerate and as a result all ADP control of respiration is lost for the period of day 3 to day 5. At 6 days, mitochondria synthesized de novo could be under partial ADP control. This RC was maximum at 7 days, and then declined to 1.0 at 10 days. The RC values, although low, are believed to be characteristic of the tissue by virtue of the fact that RC values as high as 7 can be obtained from mung bean hypocotyl mitochondria when the same isolation technique is employed (S.S. Malhotra, unpublished). The changes in RC reported in Figure 3 cannot account for the large increase in respiration rate, but nevertheless, definite parallels can be drawn: (1) When RC from 'preformed' mitochondria is dropping (0-5 days), respiration rate with succinate is increasing, and (2) both RC and respiration rate reach a peak 7 days after germination and then decline. Since ADP was unable to sufficiently control respiration, other factors must be involved. The wide difference in the utilization of substrates (Figures 1 and 2) suggests that substrate availability may, at least partially, control respiration.

It is not surprising that RC and ADP/O values follow the same trend since the calculation of an ADP/O value is dependent on control of mitochondrial respiration by ADP. Therefore, the data on ADP/O values (Figure 3) gives little information on the total phosphorylation rate in the mitochondria. The profile obtained is unlike others reported (Chapter I).

(c) ATPase Activity

The term ATPase is used here to refer to the release of Pi from ATP, and does not imply the existence of a specific enzyme. Figure 4 shows representative curves of A, phosphate concentration vs. absorbance at 313 m μ (Mozersky et al. 1966), and B, Pi liberation from ATP (ATPase activity). The profile of ATPase activity (Figure 5) parallels the respiration profile, and in certain respects, agrees with the data of Young and Varner (1959, pea cotyledon mitochondria), Young et al. (1960, pea cotyledon mitochondria), and Akazawa and Beevers (1957, castor bean cotyledon mitochondria). Maximum ATPase activity (Figure 5) occurred at 5 days and was 3 times greater than the minimum activity at 11 days. A number of factors may be responsible for the observed changes in ATPase activity with germination. ATP hydrolysis has been associated with mitochondrial swelling, but this is not necessarily a measure of mitochondrial permeability (Cereijo-Santaló, 1967). The possibility that fluctuations in ATPase activity may be brought about by changes in the concentrations of factors, such as cations, that are known to stimulate its activity, should be realized. Generally, a high ATPase activity indicates a degradation of phosphorylative function, and low P/O or ADP/O ratios would result. In some respects, the ADP/O and ATPase activity profiles do indicate this; during the early stages of germination ATPase activity is increasing and ADP/O ratios are decreasing. When the rate of ATP hydrolysis is maximum, ADP/O values are re-established and both parameters decrease together as germination continues. If comparisons can be made among different tissues, then it may be difficult to reconcile a bean cotyledon mitochondrial ATPase exhibiting a

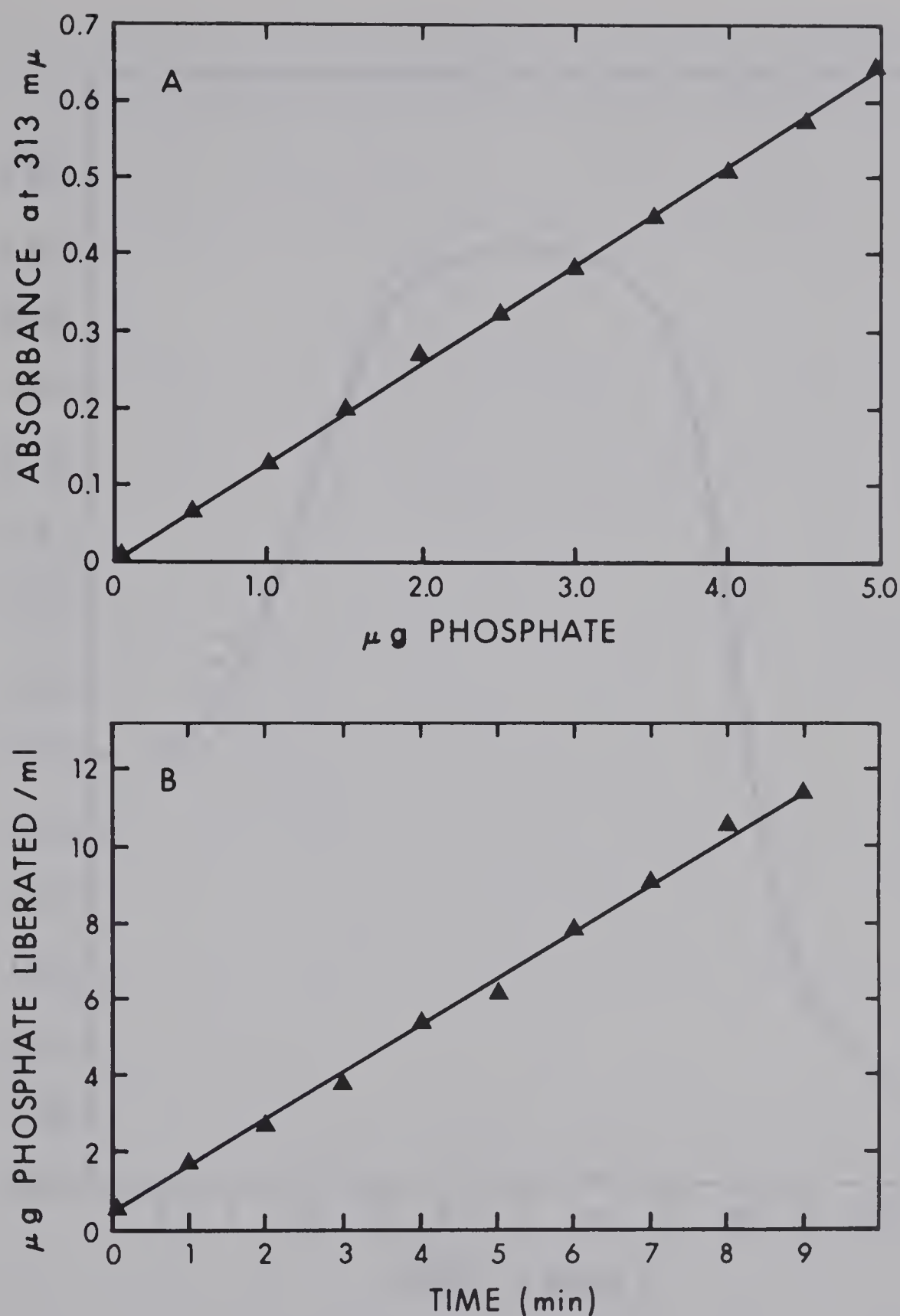


Figure 4. A. Phosphate standard curve according to the method of Mozersky et al. (1966). Phosphate concentration expressed as μg per 0.2 ml original assay solution. B. Phosphate liberated from ATP by 7 day bean cotyledon mitochondria. Assay medium: 0.3 M mannitol, 4 mM MgCl_2 , 1.0 mM TES, 2 μmoles ATP, and 1.08 mg mitochondrial protein, pH 7.2 at 24 C. Total volume was 3.1 ml.

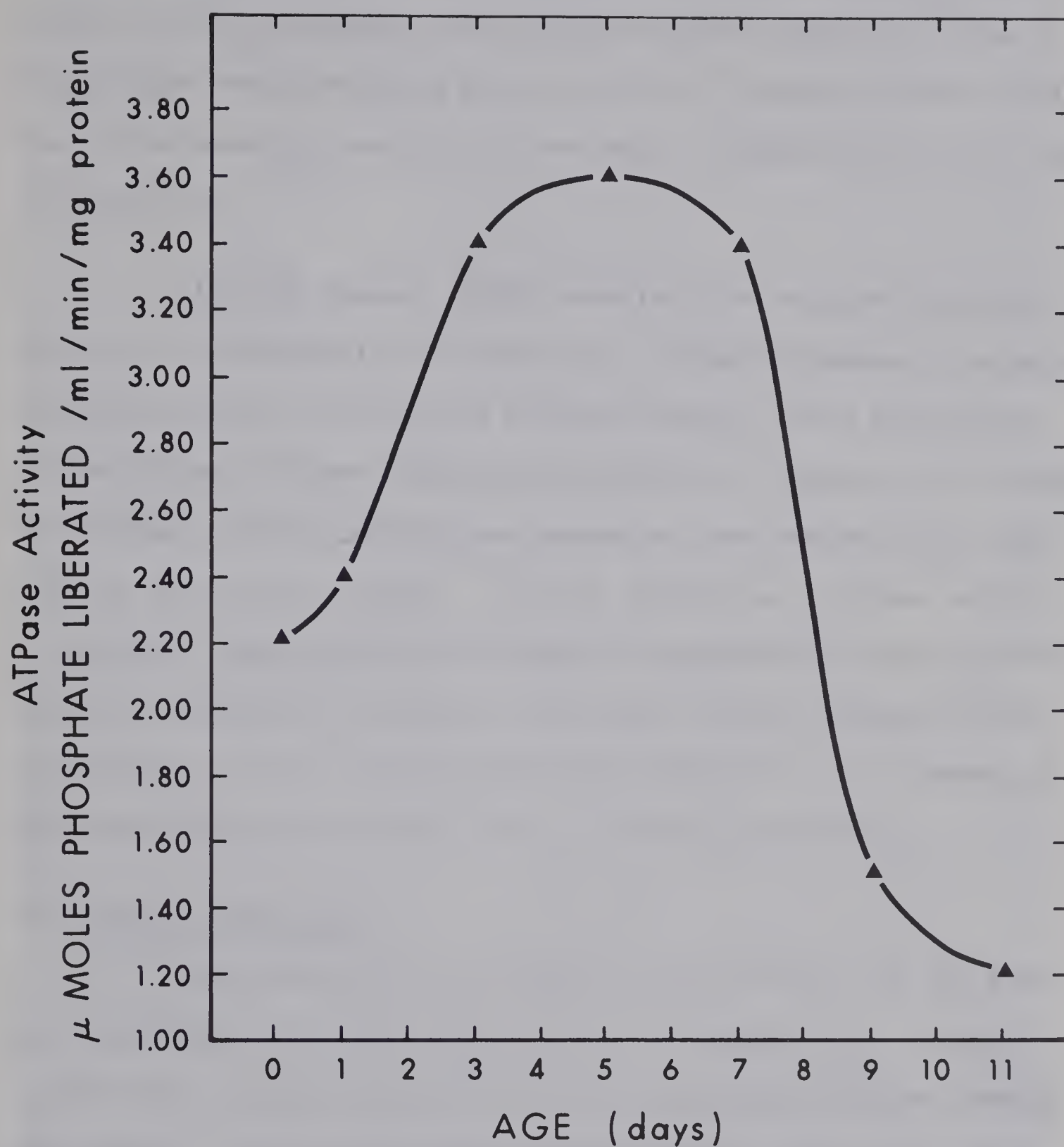


Figure 5. Rate of ATP hydrolysis by mitochondria isolated from cotyledons of germinating bean seedlings. Assay medium: 0.3 M mannitol, 4 mM MgCl_2 , 1.0 mM TES, and 2 μ moles of ATP. The pH was 7.2 and the assay temperature 24 C. Total volume was 3.1 ml.

similar age profile to those reported for mitochondrial phosphorylation in pea, peanut and soybean cotyledon mitochondria (Chapter I), when a high ATPase should result in a low P/O ratio. Increased ATPase activity may not necessarily lower RC, but can cause a sluggish state III to state IV transition.

Olson and Spencer (1968b) reported that ethylene increased the rate of mitochondrial ATP hydrolysis in bean cotyledons. The partial conformity between the ethylene evolution pattern from a particulate system (Figure 8,B) and ATPase activity (Figure 5) supports this finding; both ATPase activity and ethylene generation reach maxima at the same age and then decline together. Whether stimulation of ATPase activity by ethylene (Olson and Spencer, 1968b) is responsible for the respiration profile in Figure 1, is unknown; both ATPase activity (Figure 5) and respiration (Figure 1) profiles are very similar, but it is impossible from these data to establish a cause or effect relationship.

2. Electron Microscopy

The morphology of mitochondria isolated from 4 day and 9 day bean cotyledons is shown in Figure 6 A and B, respectively. Looped cristae (LC) are characteristic of plant mitochondria (Parsons, Bonner, and Verboon, 1965) and were quite common in these electron micrographs. Globular bodies (G) (an augmentation of looped cristae) (Van Steveninck and Jackman, 1967), and endoplasmic reticulum (ER) dotted with ribosomes were also common. A study in the electron microscope of numerous sections of mitochondria from both ages of tissue allowed the following comparisons: (1) Well defined cristae and double membranes were more prominent in mitochondria from 4 day than from 9 day old tissue, and

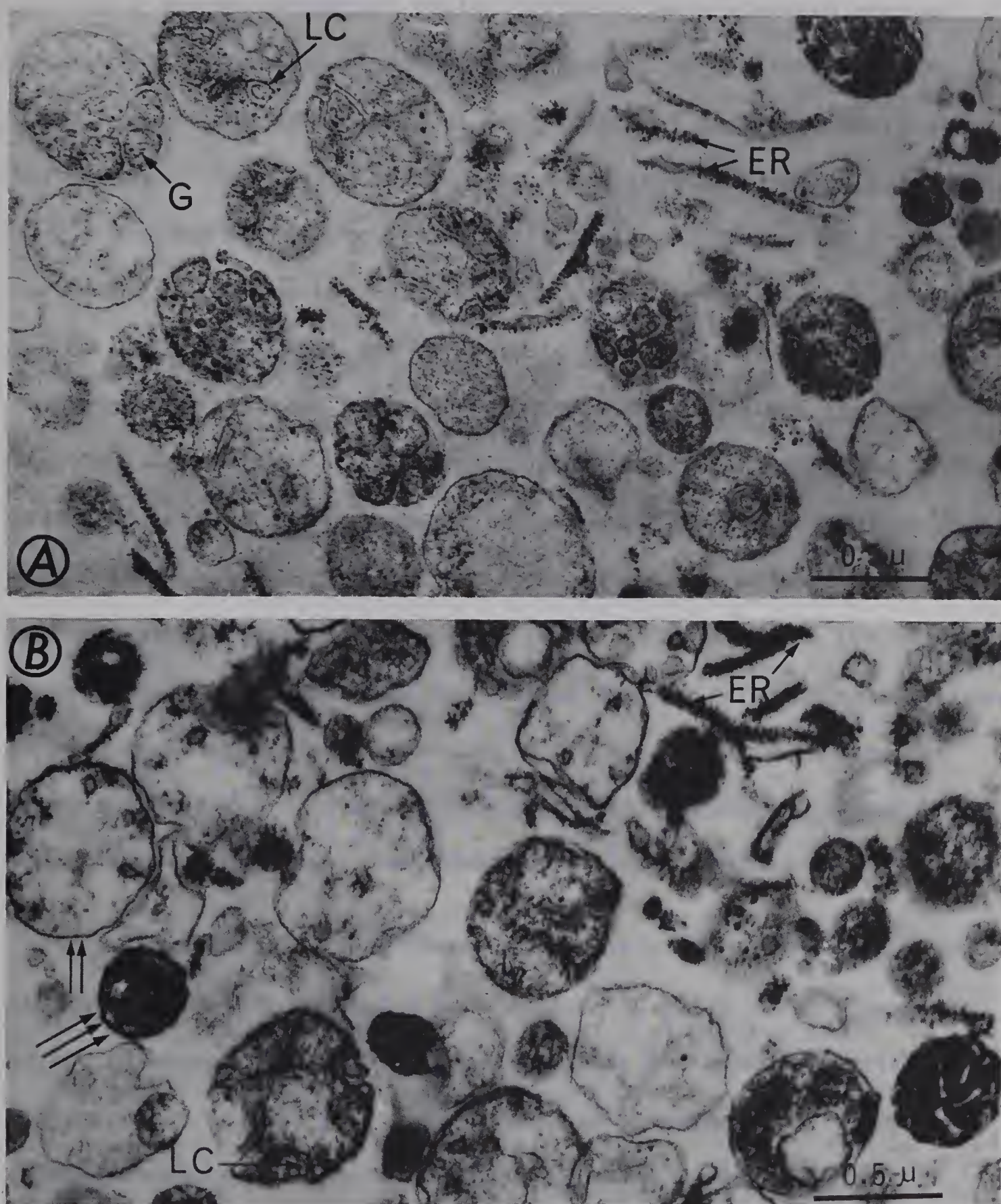


Figure 6. Electron micrographs of the mitochondrial pellets from 4 day old (A) and 9 day old bean cotyledons (B). Endoplasmic reticula (ER) are present in both ages, and looped cristae (LC) that form globular bodies (G) are common. Double arrows indicate a thin membrane, triple arrows indicate a darkened matrix.

(2) the 9 day mitochondrial pellet contained more electron opaque structures, presumably mitochondria. Many of the differences are expected, in view of the work of Öpik (1965 and 1966, bean cotyledon mitochondria) and Cherry (1963, peanut cotyledon mitochondria). Öpik (1965) suggested that the mitochondria begin disintegration by a darkening of the cristae (Figure 6), however, as Hackenbrock (1966) points out, this change can be reversible and may be controlled by the metabolic state of the mitochondrion. It is obvious from the electron micrographs, that there is much more ER contamination in the pellet from the younger tissue than in the pellet from the older tissue, and if this observation is a reality in vivo, then it undoubtedly will have a bearing on the metabolism of tissue in relation to its age. In general, it can be said that the younger mitochondria are more uniform in size and matrix density than the older mitochondria. Also, the limiting membranes of mitochondria from 9 day cotyledons appear to be much thinner, indicating that they may no longer be double.

The electron micrographs give little indication as to why mitochondria from 9 day cotyledons exhibit RC while those from 4 day do not. Parsons et al. (1965) indicate that the number of cristae have no bearing on RC, but mitochondria with an electron dense matrix and dilated intracristal spaces were found to have lower RC.

B. Association of Ethylene Evolution with Aging

Figure 7 shows representative curves of A, protein concentration vs. absorbance at 750 m μ (Lowry et al. 1951) and B, amount of ethylene vs. GC response. Ethylene evolution from whole cotyledons and from a subcellular particulate fraction of these cotyledons was studied in

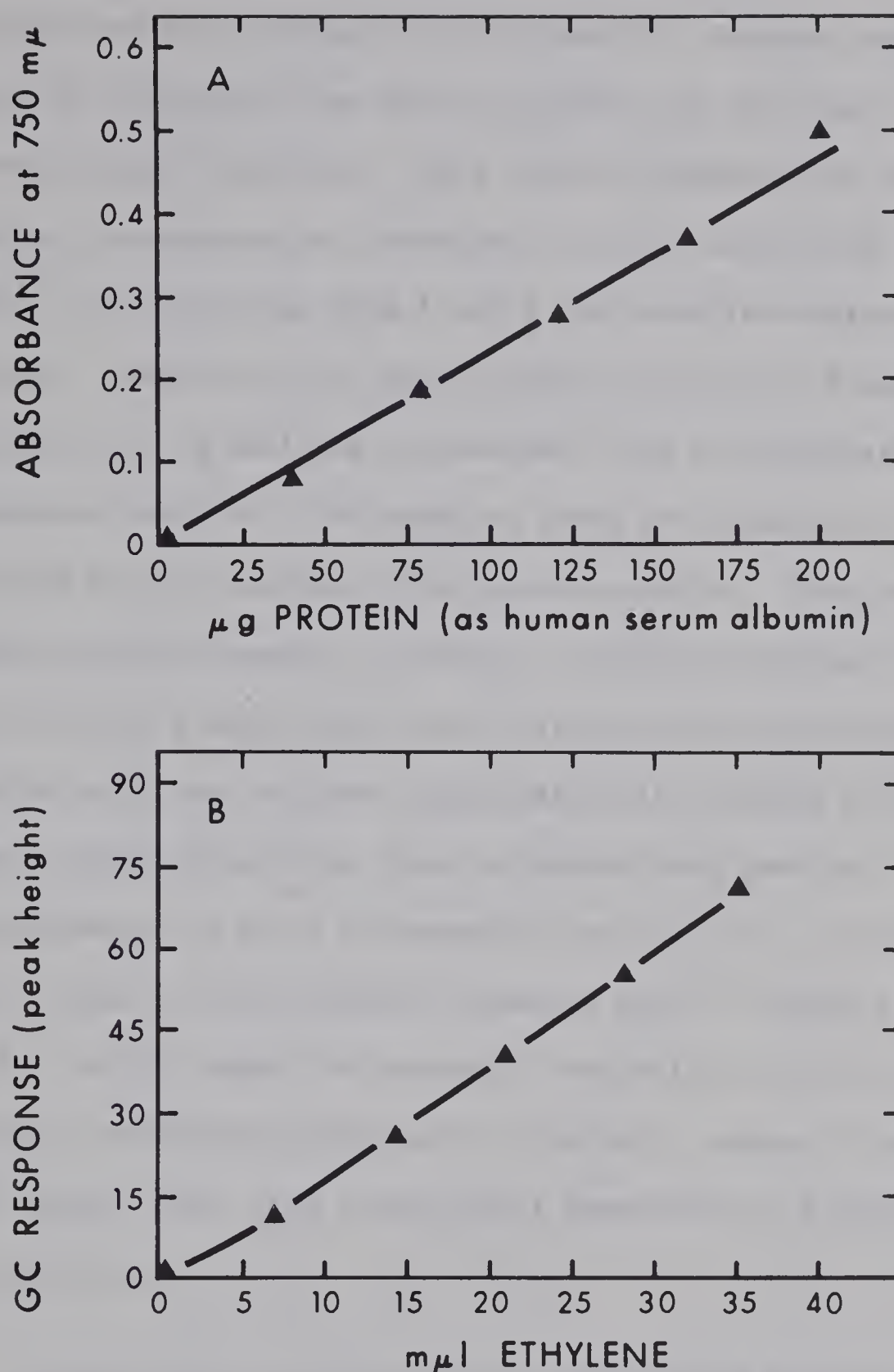


Figure 7. A. Protein standard curve (Lowry et al., 1951). Protein concentration expressed as μ g/1.0 ml original assay solution. B. Ethylene standard curve. The volatile was subjected to gas chromatography and was resolved by a column (50 cm x 6 mm ID) of activated alumina with 2½% silicone 550. He, H₂, and air flow rates were 64 cc/min, 37 cc/min, and 200 cc/min, respectively. Column temperature was approximately 20 C and detector temperature was approximately 125 C.

germinating wax beans (Figure 8, A, B, and C). Several observations were notable: (1) Evolution from whole cotyledons (A) and from a subcellular fraction (B) was concurrent. This finding suggests that in whole cotyledons, the particulate components may be a major site of ethylene evolution. (2) Evolution from A and B indicated two maxima in ethylene production. Considering the RC and ATPase profiles of Figure 3 and 5, respectively, it is exciting to speculate that the decrease in mitochondrial integrity, indicated by these two parameters, is responsible for the increase in ethylene evolution. When RC returns and ATPase activity ceases to increase, ethylene evolution drops. Loss of RC after 7 days could then indicate further mitochondrial degradation with the resultant augmentation in ethylene synthesis. (3) When ethylene generation from the subcellular particulate fraction (B) was converted to $\mu\text{l/g}$ mitochondrial protein (C), the profile changed. After 7 days, ethylene formation did not decrease in C as it did in B. On this basis the system(s) responsible for ethylene biosynthesis become more prominent in the later stages of germination than in earlier ones, even though total generation of ethylene and total protein decrease.

Sonication of the subcellular particulate fraction before collection resulted in a 10 fold increase in ethylene, but generation of the volatile in relation to age was similar to the unsonicated sample. When ethylene evolution from whole cotyledons was put on a per g dry weight basis, a similar curve to that of Figure 8, C was obtained.

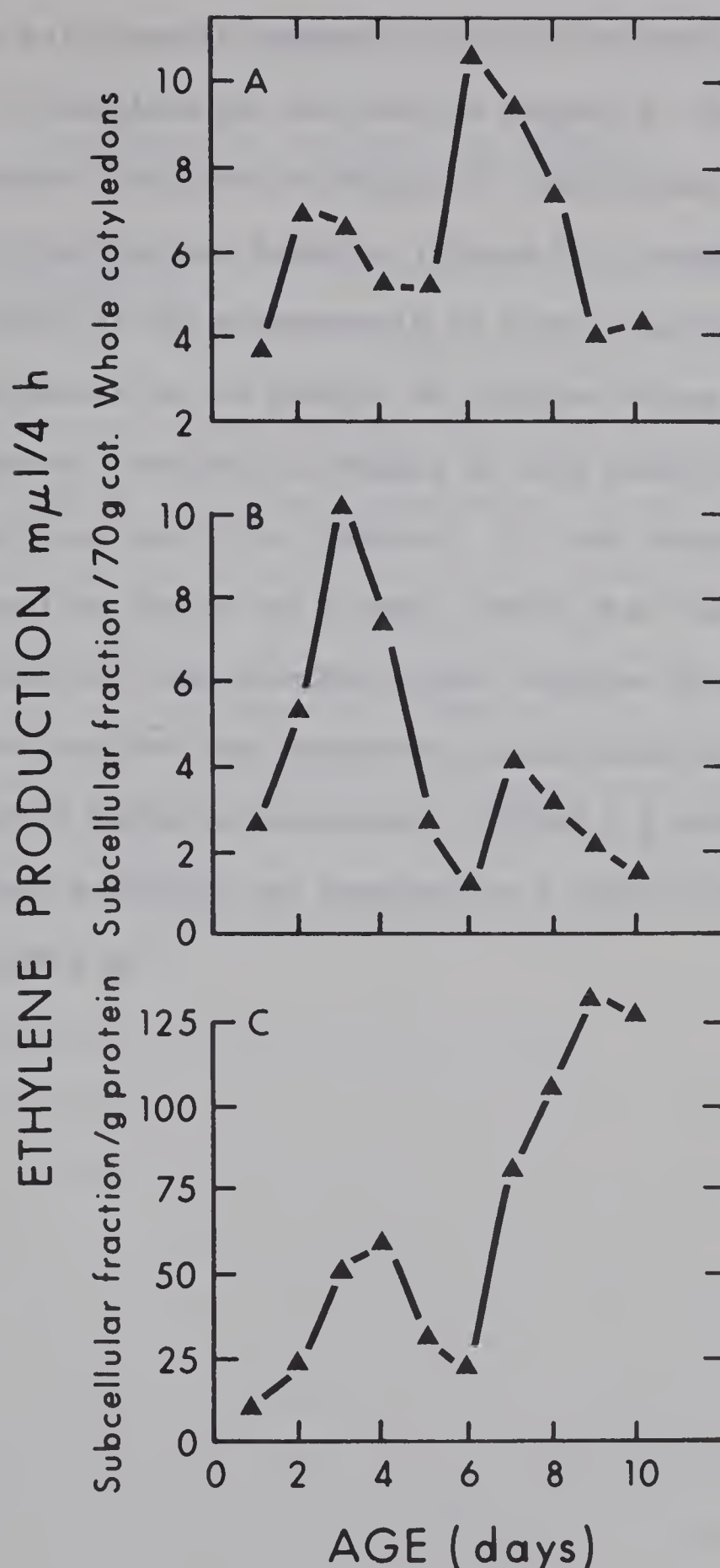


Figure 8. Ethylene production by 30 g of whole bean cotyledons (A), and by a subcellular particulate fraction from bean cotyledons, expressed as per 70 g whole cotyledons (B) and as per 1 g protein (C). The subcellular particulate fraction was suspended in water, TES (10 mM), and ATP (1.5 mM), and ethylene collected at pH 7.0. Protein was determined by the Lowry method (Lowry et al., 1951).

The relationship between propionate metabolism and conversion of β -alanine to ethylene was outlined in Chapter I; the similarity between propionate respiration (Figure 2) and ethylene evolution/g protein from a particulate fraction (Figure 8, C) suggests that increased ability of the mitochondria to oxidize propionate after 7 days may be a factor in the control of ethylene biosynthesis. However, malonate supported respiration (Figure 2) also exhibited a profile similar to ethylene evolution (Figure 8, C), and enzymes involved in malonate metabolism (Hatch and Stumpf, 1962b) that leads to acetyl CoA production, have not been associated with ethylene biosynthesis. It should also be recalled that propionate respiration rates were determined with a washed mitochondrial ($10,000 \times g$ fraction) suspension, whereas ethylene evolution was examined on a subcellular particulate fraction ($32,000 \times g$).

CHAPTER IV

CONVERSION OF β -ALANINE TO ETHYLENE

A. Cofactor Stimulation of Ethylene Production from a Lyophilized Particulate Fraction

Table 1 shows the stimulation in ethylene production obtained when the cofactors necessary for the conversion of β -alanine to ethylene (Thompson and Spencer, 1966) were added to a suspension of 0.5 g lyophilized particulate fraction. From both experiments, it can be seen that the cofactors caused a significant increase in ethylene production, especially on the 2-4 h collection. Cofactors alone gave no ethylene. The larger stimulation on the 2-4 h collection suggests that cofactor(s) or substrate(s), normally used up on the 0-2 h collection, were replenished by the complement of cofactors added.

B. Solubilization of the Particulate Fraction

Table 2 compares five treatments used to solubilize the proteins of a 3½ day old bean cotyledon subcellular particulate fraction that was capable of converting β -alanine to ethylene. Sonication solubilized a great deal of protein, and very large amounts of the volatile were produced if collection on this soluble fraction was commenced immediately after sonication and centrifugation. The sonicated sample in Table 2 was centrifuged and the supernatant fraction was then freeze-dried to remove any ethylene formed during sonication. Since the solubilization treatment constitutes a purification step, the important criterion is the amount of ethylene per mg protein; Triton X-100 (0.4%) was the preferred agent for solubilization. Triton X-100 at the 1% level solubilized more protein, but the higher level of detergent was inhibitory to ethylene production.

Table 1. Cofactor stimulation of ethylene production by a lyophilized particulate fraction (0.5 g).

Experiment	Sample	Collection period			
		0-2 h		2-4 h	
		m μ l ethylene	% stimulation with cofactors over powder alone	m μ l ethylene	% stimulation with cofactors over powder alone
1	Powder alone	18.6	0	6.8	0
	Powder + co- factors	25.6	+38	15.5	+128
2	Powder alone	26.4	0	10.2	0
	Powder + co- factors	31.5	+19	21.3	+109

Cofactors were β -alanine (50 mM), malonate (50 mM), α -KG (50 mM), ATP (1.5 mM), CoA (0.17 mM), TPP (2.0 mM), MgSO_4 (1.0 mM), and pyridoxal phosphate (0.5 mM). Collection was at pH 7.0 and room temperature.

Table 2. Solubilization of a particulate fraction capable of converting β -alanine to ethylene.

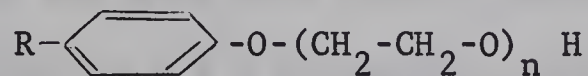
Solubilization treatment	mg protein solubilized from 500 mg dry powder	m μ l ethylene from soluble protein (0-2 h)	m μ l ethylene per mg protein	Percentage [*] ethylene activity solubilized
Sonication	60	10	0.16	34
Osmotic rupture	17	2	0.12	7
1% Tween 80	39	10	0.26	31
1% Triton X-100	64	9	0.14	28
0.4% Triton X-100	35	12	0.33	40

* The theoretical amount of ethylene was obtained from a collection on 500 mg lyophilized particulate fraction.

All reaction mixtures contained β -alanine (50 mM), malonate (50 mM), α -KG (50 mM), ATP (1.5 mM), CoA (0.17 mM), TPP (2.0 mM), MgSO_4 (1.0 mM), and pyridoxal phosphate (0.5 mM). pH 7.0, final volume 20 ml.

An interesting observation is that 0.4% Triton X-100 solubilized twice as much protein as buffer alone (osmotic rupture), but there was 6 times more ethylene evolved from the detergent solubilized protein. This is possibly an indication that the enzyme(s) responsible for ethylene biosynthesis may be partially associated with insoluble membrane protein.

The use of detergents for the solubilization of enzymes from tissues has become quite common. Triton X-100 is a nonionic liquid with the following formula (Enyeart, 1967):



n = Poisson distribution $\text{R} = \text{CH}_3-(\text{CH}_2)_7-$

Octylphenoxypolyethoxyethanol

Like most detergents, Triton X-100 solubilizes by use of electrostatic and Van der Waals forces (Putnam, 1948).

The lyophilized particulate fraction was found to contain approximately 100 mg protein, and therefore, the 0.4% Triton X-100 treatment solubilized 29% of the protein. The routine solubilization procedure involved two treatments with Triton X-100 (Chapter II), and this resulted in twice as much soluble protein with no decrease in specific activity. The soluble protein obtained in 60 ml after gel filtration on Sephadex G-25, was divided among 4 samples; the mean protein level per collection was 82 mg, the standard deviation was 9 mg.

Samples of lyophilized subcellular fraction and soluble enzyme system, each containing β -alanine (50 mM), malonate (50 mM), and α -KG (50 mM), NADH (0.83 mM), DTT (0.7 mM) and standard amounts of ATP, TPP, MgSO_4 , and pyridoxal phosphate, were shown to evolve 0.26 and 0.39 μl of ethylene/mg protein, respectively.

C. Levels of NADPH and NADH, and Ethylene Biosynthesis

The reduction of malonic semialdehyde (MSA) to β -hydroxypropionate is a required reaction in the conversion of β -alanine to ethylene as proposed by Thompson and Spencer (1966), (Chapter I). However, in their partially purified enzyme system from bean cotyledons, the conversion occurred in the absence of reducing agents. Figure 9 shows the amount of ethylene evolved, over a control with no reduced pyridine nucleotides added, from the soluble system when various levels of NADPH and NADH were added. Both compounds greatly stimulated ethylene production on a 2 h collection, but NADH achieved the same degree of stimulation at one half the concentration of NADPH; maximum stimulation for both reduced pyridine nucleotides was approximately 190%. Comparisons were only made among samples from the same enzyme preparation. In contrast to the cofactors tested in Table 1, NADH and NADPH had their maximum effect on the 0-2 h collection. Evolution on the 2-4 h collection was always greater with NADPH (256% stimulation (1.12 mM) as opposed to only 160% from NADH). This may indicate that the large stimulation by NADH on the 0-2 h collection has made another factor limiting or, is itself limiting. When the linear portions of the two curves are considered, it can be calculated that 30,000 molecules of NADPH are required to stimulate the synthesis of 1 additional molecule of ethylene. This large ratio may indicate destruction of the nucleotide. NADH had no effect on ethylene production from the lyophilized subcellular particulate fraction per se.

Further experiments showed that neither β -alanine, α -KG, nor malonate was limiting on the 2-4 h collection. However, when 12.5 mg NADH were added after a 2 h collection that initially contained 12.5 mg

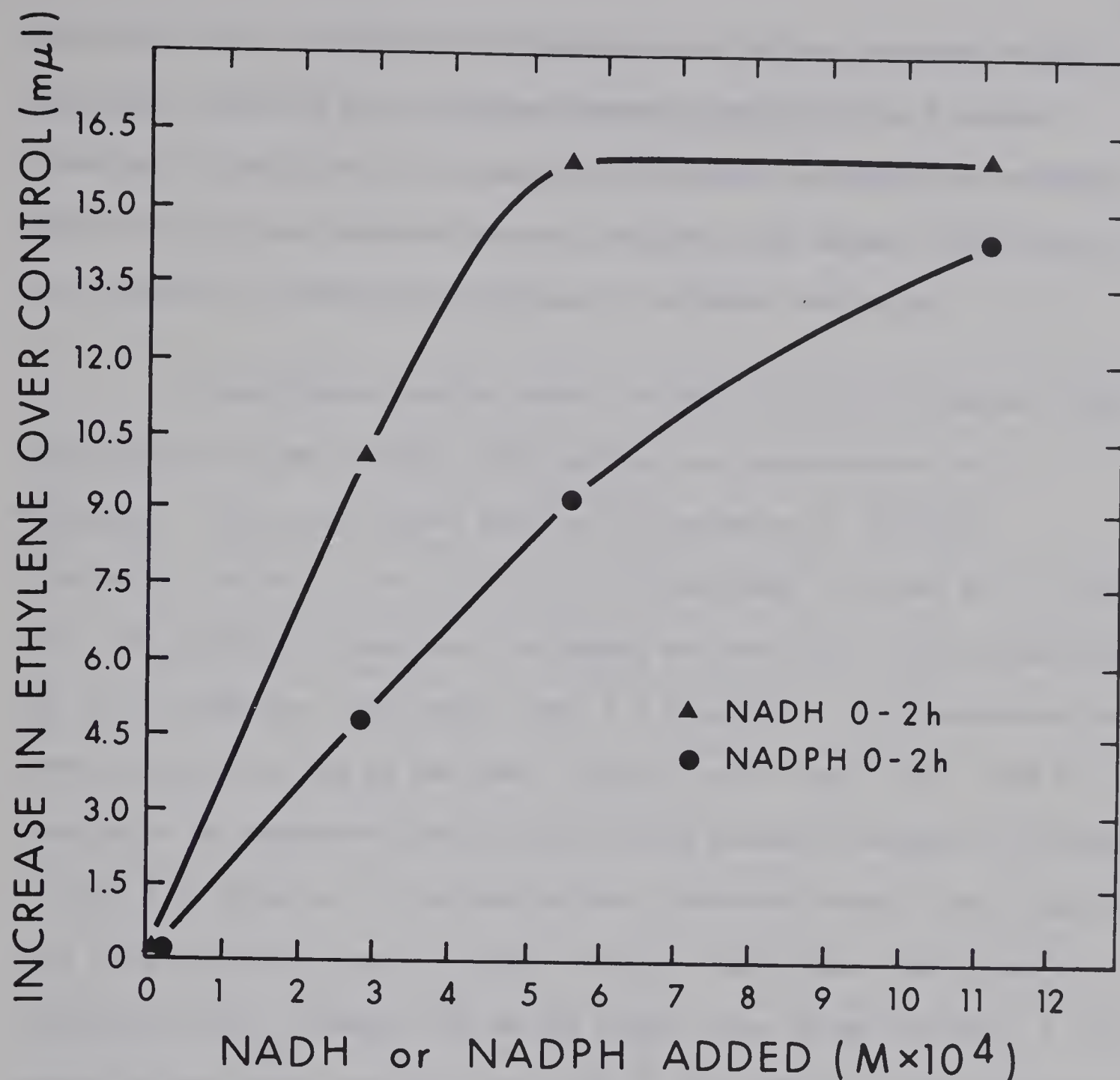


Figure 9. Effects of various levels of NADH and NADPH on ethylene production by a solubilized particulate fraction from wax bean cotyledons. Ethylene obtained in the absence of any reduced pyridine nucleotide (control) was subtracted from that obtained when reduced pyridine nucleotide was added. Each collection was 50 mM in β -alanine, 50 mM in malonate, 50 mM in α KG, 1.5 mM in ATP, 2.0 mM in TPP, 1.0 mM in MgSO_4 , 0.5 mM in pyridoxal phosphate, pH 7.0. Final volume of 20 ml contained approximately 82 mg of protein.

NADH (0.83 mM), an additional stimulation of 88% was achieved in one experiment and 159% on a different enzyme preparation in a second experiment. Addition of 3.4 μ moles of rotenone, an inhibitor of NADH oxidase in certain systems (Ernster, Dallner, and Azzone, 1963), did not result in a significant increase in ethylene evolution.

A spectrophotometric assay for NADH (Ciotti and Kaplan, 1957) utilized the enzyme alcohol dehydrogenase and acetaldehyde as a substrate. The assay showed that in the presence of cofactors (β -alanine, malonate, and α -KG all at 50 mM; NADH, 0.83 mM; TES, 10 mM; ATP, TPP, pyridoxal phosphate, and MgSO_4 as usual; pH 7.0), approximately 25% of the NADH was 'destroyed' over a 4 h period. This percentage was even greater when the pH was made slightly acidic (pH 6.8). This was considered an important factor since the pH normally dropped to between 6.7 and 6.8, after a 4 h collection that contained protein and cofactors. Once this destruction was realized, further experiments were done at increased buffer strength (50 mM TES rather than 10 mM) and pH 7.2. It cannot be said with certainty how much of the NADH was destroyed and how much was oxidized to NAD^+ . In any case, use of alcohol dehydrogenase in the collection medium in an attempt to regenerate NADH from NAD^+ , and thus provide an ample supply for reduction of MSA, was unsuccessful.

D. Effect of Dithiothreitol on Ethylene Biosynthesis in the Crude and Soluble Enzyme Systems

Because dithiothreitol (DTT) has a low redox potential, it is capable of maintaining monothiols in the reduced state (sulfhydryl protector) (Cleland, 1964). When this compound was added (final concentration 9.7 mM) to a suspension of 0.5 g lyophilized particulate fraction that was 50 mM β -alanine, 50 mM malonate, 50 mM α -KG,

10 mM TES, and contained standard amounts of ATP, TPP, MgSO_4 and pyridoxal phosphate (pH 7.0), there was no augmentation of evolved ethylene. However, when the same amount of the dithiol was added to a collection that contained, in addition to the above factors, NADPH (0.56 mM, reported above to give no stimulation), a 40% stimulation in evolution of the volatile was observed. Perhaps NADPH is rapidly destroyed when added to the crude system, and DTT is able to maintain the nucleotide in a form that can be used by the system responsible for the biosynthesis of ethylene. DTT (9.7 mM) stimulated ethylene production from the soluble system (all cofactors and substrates present) in the absence of NADPH; this increase, however, was 62% compared with 115% for NADPH (0.56 mM). The presence of both compounds did not result in a significant increase in the amount of the volatile over NADPH alone. These data suggest that either the nucleotide is not limiting in the crude system or that it is prevented, possibly by 'destruction', from exhibiting its stimulatory effect. It was later discovered that DTT, in the absence of ^{NADH and} substrate (cofactors present), could cause a significant stimulation in the evolution of ethylene from the soluble system. Perhaps the dithiol, through its protective action, was either promoting the conversion to ethylene of enzyme bound intermediates, or its action may have been through an unknown mechanism. Indeed, evidence has accumulated during the course of this investigation (see subsequent chapters) to suggest that compounds, capable of being converted to ethylene, are present (despite 'extensive dialysis') in the soluble system.

E. Ethylene Biosynthesis as a Function of pH

Since only four samples could be investigated at one time, several experiments were carried out and integrated to obtain the curves

in Figure 10. On both 0-2 and 2-4 h collections, ethylene producing activity reached a peak at pH 7 and then declined. However, if pH was raised further, activity again increased in both collection periods. Ethylene evolution on the 0-2 h collection continued to rise up to pH 12. Evolution on the 2-4 h collection reached a second peak at pH 8.5, and then rapidly declined. Although the two curves appear to be contradictory, there may be a simple explanation; the large amounts of ethylene evolved on the 0-2 h collection at pH 9 and above could cause a factor to become limiting for the subsequent collection period. It is possible that the 0-2 and 2-4 h profiles could be identical at the higher pH values, if there was no limiting factor on the 2-4 h collection.

It is significant that at pH 9 the cofactors alone gave no ethylene. If protein alone was allowed to be collected 2 h at pH 9 and then cofactors added, a stimulation of ethylene evolution of several hundred percent was achieved. This finding suggests that the ethylene evolved at pH 9 may be enzymic. If the ethylene evolved at pH 10 and above is enzymic, it is unlikely that it would be of significance in vivo. A maximum at pH 7.0 simulates a more conventional pH dependence: Mapson and Wardale (1967) found an enzyme system from cauliflower florets^{that} converted methional to ethylene maximally at pH 6.6, Shimokawa and Kasai (1967b) found pH 6.0 was optimum for the formation of ethylene from pyruvate by a subcellular fraction from apple tissue, and Yang (1967) found a pH range from 7.3-8.3 was optimum for the conversion of methional to ethylene by horseradish peroxidase.

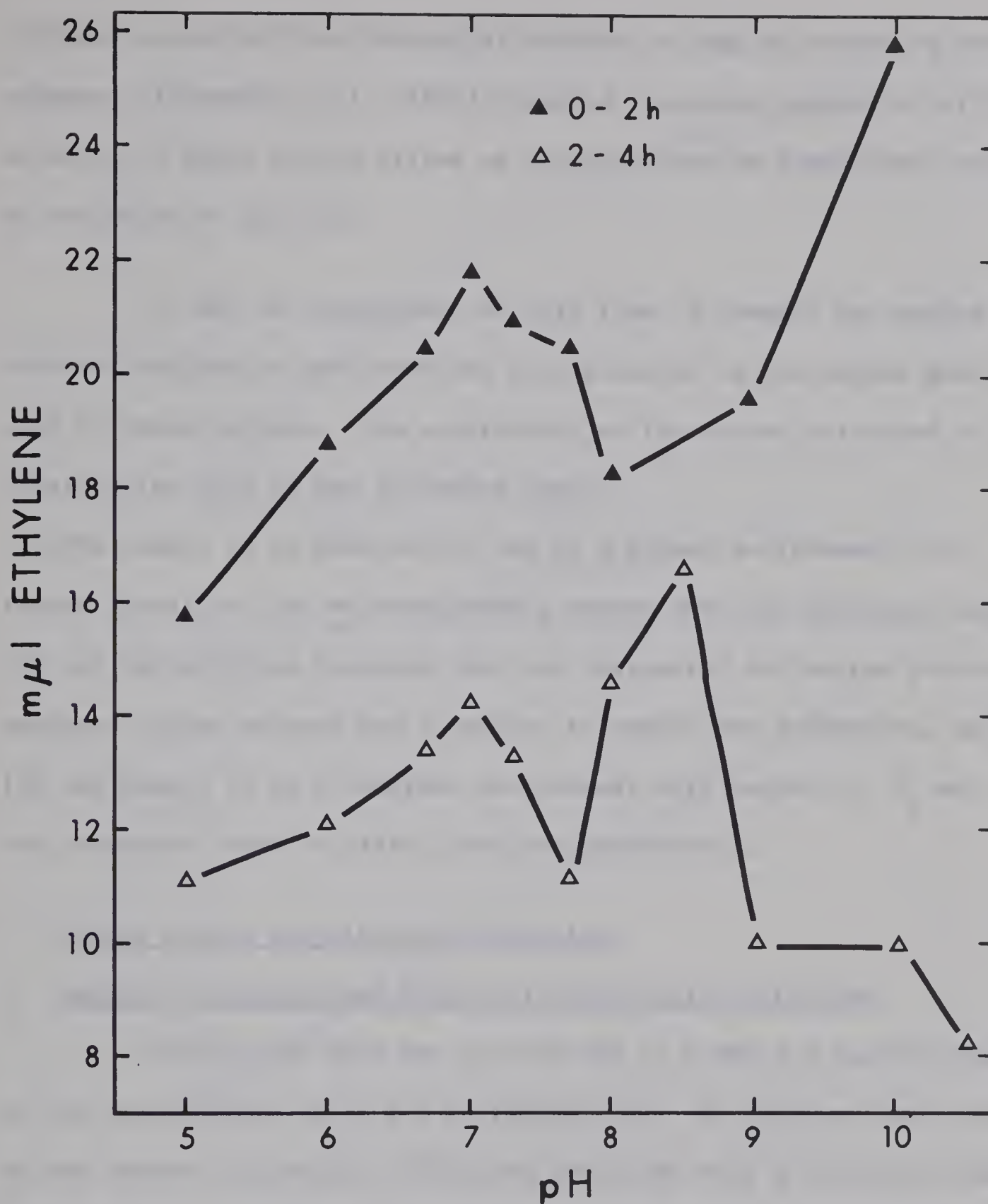


Figure 10. Effect of pH on ethylene biosynthesis by a soluble enzyme system. Each assay contained β -alanine (50 mM), malonate (50 mM), α -KG (50 mM), ATP (1.5 mM), TPP (2.0 mM), pyridoxal phosphate (0.5 mM), MgSO_4 (1.0 mM), and TES (10 mM). Approximately 82 mg of protein were in a final volume of 20 ml.

Ethylene evolution from biological systems at high pH values is not unknown; Lieberman et al. (1966) reported increased production of the volatile in apple tissue slices up to pH 9.5 and no significant drop in evolution to pH 10.5.

It may be appropriate at this time to compare the method of ethylene collection developed for this research to the method most often used by other workers. The superiority of the method described in this dissertation lies in the following facts:

- (1) The sample to be analyzed is not in a closed environment, but rather 'purified' air is continuously passed over the agitated sample,
- (2) all the ethylene produced over the designated collection period is analyzed; other methods use a syringe to sample the atmosphere, and
- (3) the sample is in a constant environment with respect to O_2 and CO_2 , two compounds known to affect ethylene biosynthesis.

F. Enzyme Levels and Ethylene Production

1. Amounts of Lyophilized Subcellular Particulate Fraction

Collections were set up with ATP (1.5 mM) and various weights of the crude powder up to 0.5 g (Figure 11B). No ethylene was produced in the absence of protein. Ethylene evolution over a 4 h period was proportional to enzyme concentration over the range investigated. In a separate experiment, 1.0 g of lyophilized powder gave no more ethylene than did 0.5 g.

2. Levels of Soluble Protein

Figure 11A shows ethylene production under standard conditions over a 4 h period at different enzyme concentrations. Production was

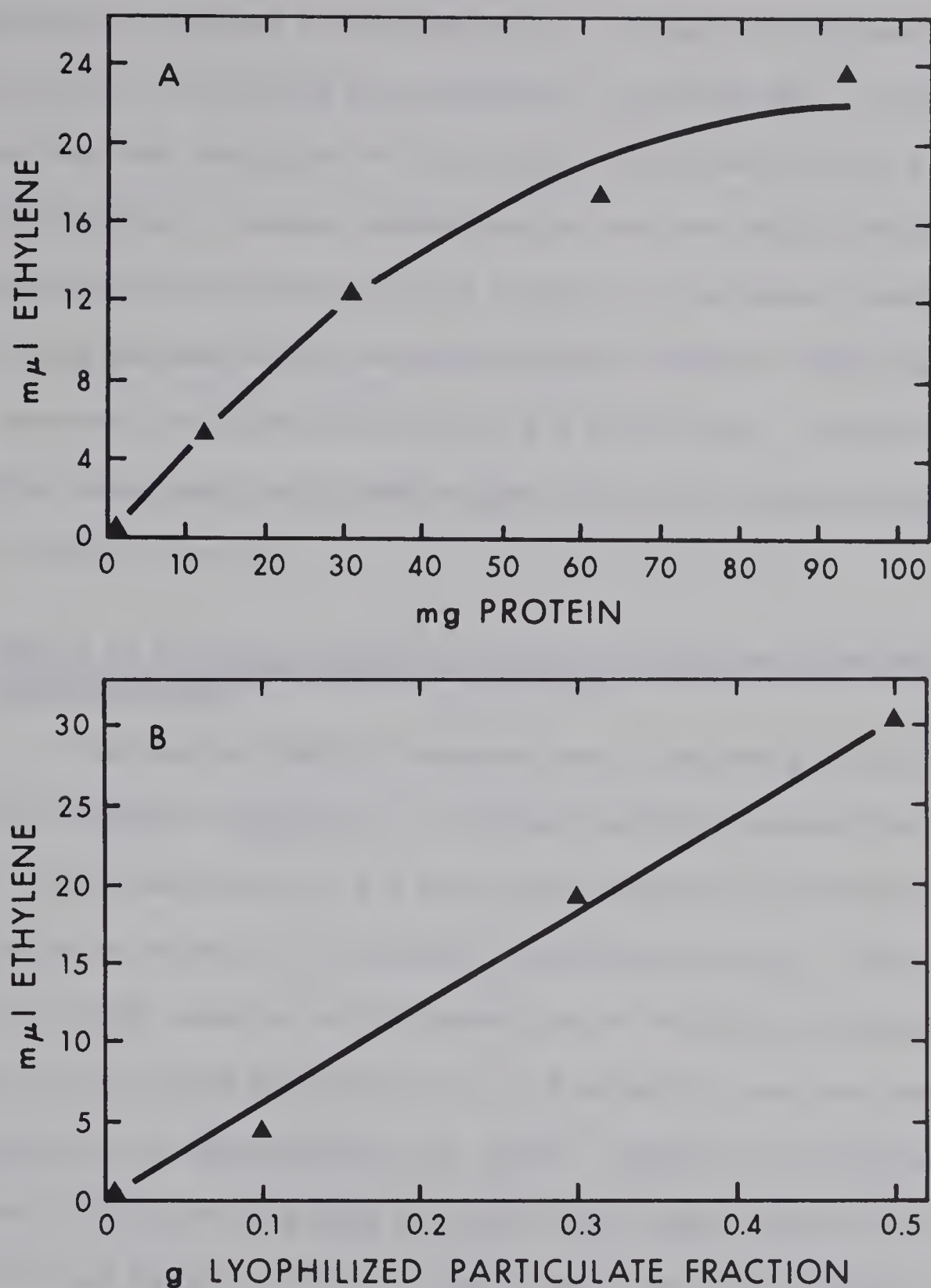


Figure 11. A. Ethylene production over a 4 h period at various concentrations of soluble enzyme solution. Reaction mixtures contained β -alanine (50 mM), ATP (1.5 mM), TPP (2.0 mM), MgSO_4 (1.0 mM), pyridoxal phosphate (0.5 mM), NADH (0.83 mM), and TES (50 mM), pH 7.2. Total volume was 20 ml.

B. Ethylene production over a 4 h period at various concentrations of lyophilized subcellular particulate fraction. Reaction mixtures contained β -alanine (50 mM), ATP (1.5 mM), and TES (50 mM), pH 7.0. Total volume was 20 ml.

proportional to enzyme concentration up to 15 μ l of ethylene and 45 mg protein/20 ml, but beyond this production levelled off. It was interesting that evolution of the volatile over the initial 2 h period was proportional to enzyme concentration over the entire range investigated, but evolution for the second 2 h increased linearly only up to 30 mg protein/20 ml, and then dropped sharply. This suggests that some factor(s) are limiting on the 2-4 h collection. Previously described experiments with NADH suggest that this compound may be one of the limiting factors.

G. Effects of Heating, Arsenite, or Urea on Ethylene Biosynthesis in a Soluble System

The data in Table 3 indicate that a majority of the ethylene evolved originates enzymically. Heating inhibited production by almost 100%. A 50% inhibition by 8 M urea could indicate an essential association of subunits for activity (Braunitzer et al., 1964). Meheriuk (1965) reported a 90% inhibition of ethylene evolution from a tomato particulate fraction with 0.1 M arsenite, but the inhibition obtained here is considerably less (63%). However the presence of DTT in these collections may have protected the enzyme from arsenite (Hochster and Quastel, 1963). Inhibition by urea and arsenite was considerably less during the 2-4 h reaction period, and is because of a decrease in ethylene production by the control after 2 h. Because of the low production of the volatile on the 0-2 h collection from the inhibitor treated sample, there is less likelihood of a cofactor becoming limiting in the second 2 h collection. The data reported previously for NADPH (Figure 9) indicate that the level of the

Table 3. Effect of heating, arsenite, or urea on ethylene biosynthesis by a soluble system.

Sample treatment	Collection period			
	0-2 h		2-4 h	
	m μ l ethylene	% difference from no treatment	m μ l ethylene	% difference from no treatment
None	17.9	0	10.7	0
Heated	0.6	-97	0	-100
Urea (8 M)	9.0	-50	8.2	-23
Arsenite (0.1 M)	6.6	-63	7.4	-31

Each sample was 50 mM in β -alanine, 50 mM in malonate, 50 mM in α -KG, 0.28 mM in NADPH, 9.7 mM in DTT, 1.5 mM in ATP, 2.0 mM in TPP, 1.0 mM in MgSO_4 , 0.5 mM in pyridoxal phosphate, and 10 mM in TES, pH 7.0. Approximately 82 mg of protein were in a volume of 20 ml.

nucleotide used in these inhibitor experiments could be limiting on both the 0-2 and 2-4 h collections.

H. Effects of Malonate, β -alanine, and α -ketoglutarate on Ethylene Production

1. Malonate Levels

The pathway outlined by Thompson and Spencer (1966) for the conversion of β -alanine to ethylene by a non solubilized particulate system involved malonate in a side path. They reported that 50 mM malonate stimulated ethylene production from β -alanine by 83% and suggested a precursor role for this acid (Thompson and Spencer, 1967). Reduction of malonate to malonic semialdehyde (MSA) links the former with the pathway converting β -alanine to ethylene (Chapter I). Ethylene evolution for the first 2 h period was proportional to malonate concentration until the acid reached 35 mM (Figure 12). Production of the volatile on the 2-4 h collection at low malonate levels was greater than on the 0-2 h collection, but evolution began to decrease at concentrations of malonate greater than 10 mM. This drop could be attributed to either inhibitor accumulation or the depletion of an essential factor. NADH was not added to these samples and therefore its depletion would not be responsible for the decrease in evolution of the volatile on the 2-4 h sampling. The largest stimulation (at 50 mM malonate) on the 0-2 h collection was 204% over a control with no malonate, whereas maximum ethylene was evolved at 10 mM malonate on the 2-4 h collection and was 145% over control. It appears from Figure 12 that malonate had a precursor role, as speculated by Thompson and Spencer (1966), but this would be impossible to confirm without labelled experiments (see later section). M.J. Coon (personal communication)

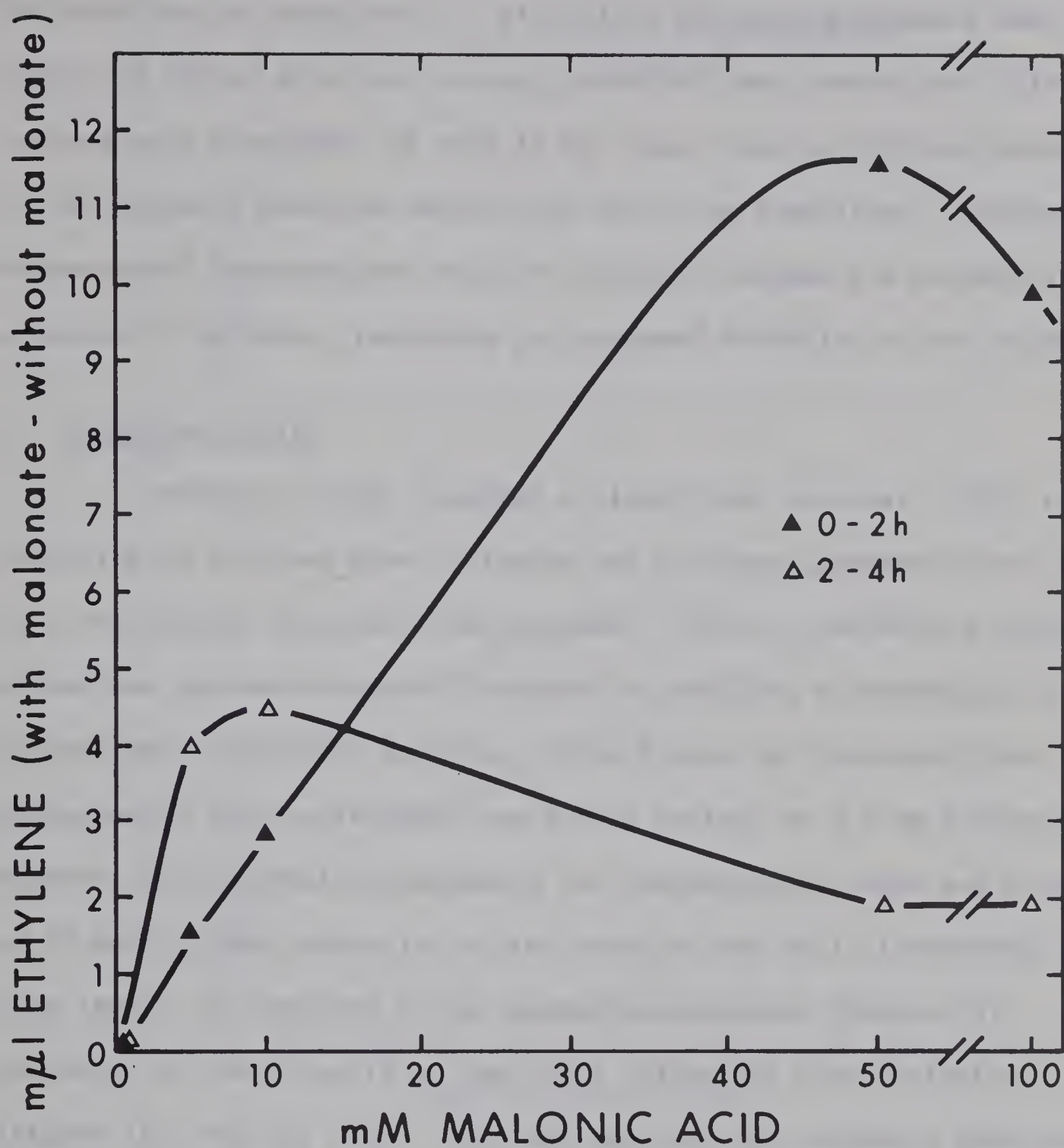


Figure 12. Ethylene biosynthesis as a function of malonic acid concentration. Reaction mixtures were 1.5 mM in ATP, 2.0 mM in TPP, 1.0 mM in MgSO_4 , 0.5 mM in pyridoxal phosphate, and 10 mM in TES, pH 7.0. Approximately 82 mg of protein were in a final volume of 20 ml.

suggested that the energy necessary for the conversion of malonate to MSA could not be supplied by a biological system and malonate must exert its effect as an end product inhibitor, and prevent the 'flow' of carbons away from MSA. If this is the case, then an ethylene precursor in the malonate reaction mixture has yet to be identified. Perhaps enzyme bound intermediates could be 'forced' through the pathway by the presence of malonate, resulting in increased evolution of the volatile.

2. β -alanine Levels

Meheriuk (1965) reported a significant increase (150%) in evolution of ethylene when β -alanine and pyridoxal phosphate were added to a subcellular fraction from tomatoes. Figure 13 shows the effects of various concentrations of β -alanine on ethylene biosynthesis by the solubilized particulate fraction. This Figure (A) indicates that evolution on both collections was almost maximal at 0.5 mM β -alanine. However, in a separate experiment, the concentration range was extended to 90 mM (B), and production of the volatile was still increasing at this level. In contrast to the malonate experiment (Figure 12), evolution of the volatile on the 2-4 h collection with β -alanine (Figure 13), did not drop. This may indicate that malonate does not actually function as a precursor but β -alanine does; there is no large drop on the 2-4 h β -alanine collections because substrate is not limiting as it would be in the malonate experiments if malonate itself were not a substrate. Thompson and Spencer (1967) were able to show that ethylene- ^{14}C could be derived from β -alanine- ^{14}C in the presence of an enzyme powder prepared from a subcellular particulate fraction of bean cotyledons.

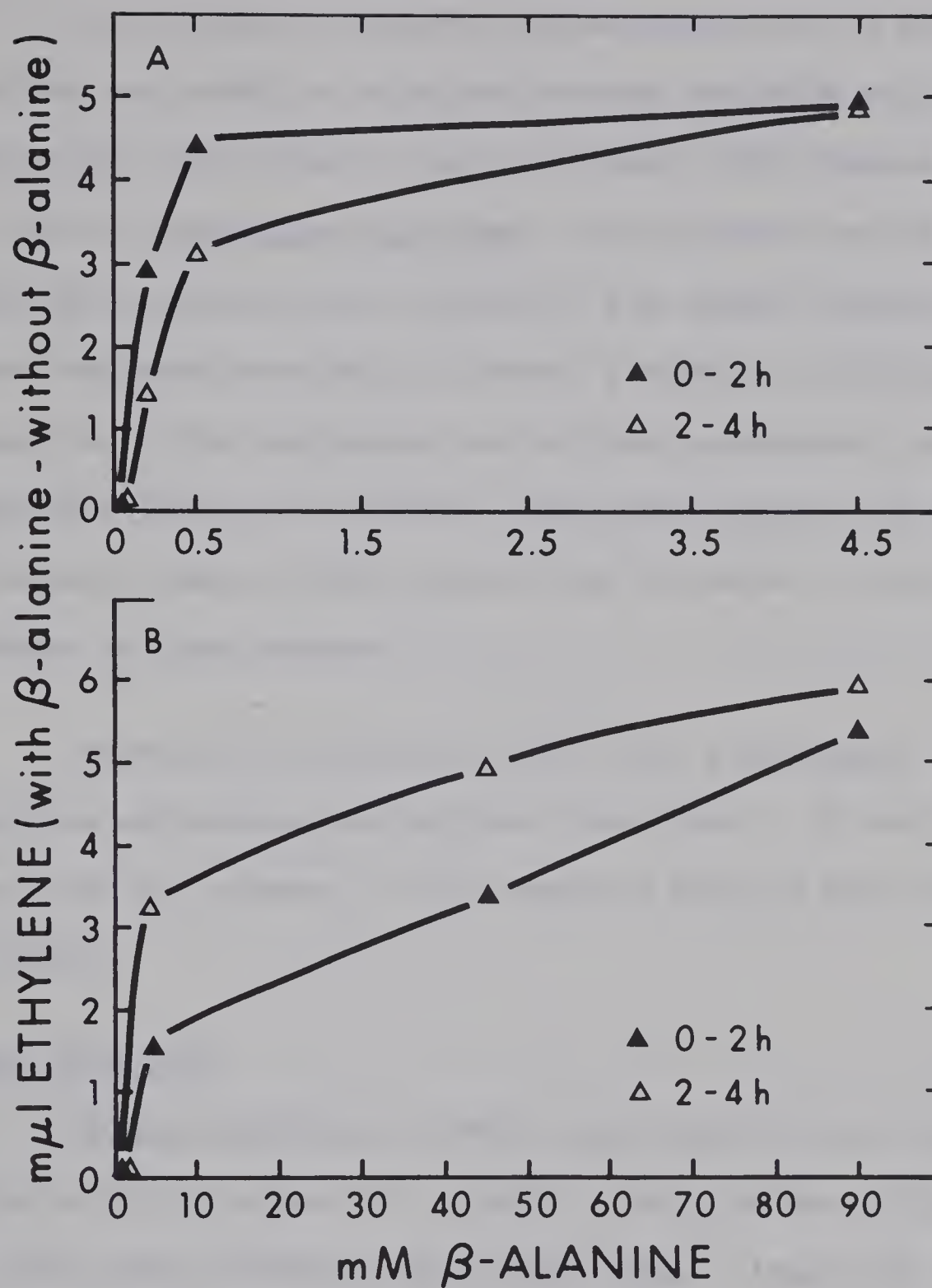


Figure 13. Ethylene biosynthesis as a function of β -alanine concentration. Reaction mixtures were 1.5 mM in ATP, 0.17 mM in CoA, 2.0 mM in TPP, 1.0 mM in $MgSO_4$, 0.5 mM in pyridoxal phosphate, and 10 mM in TES, pH 7.0. Collections contained approximately 82 mg protein. Final volume was 20 ml.

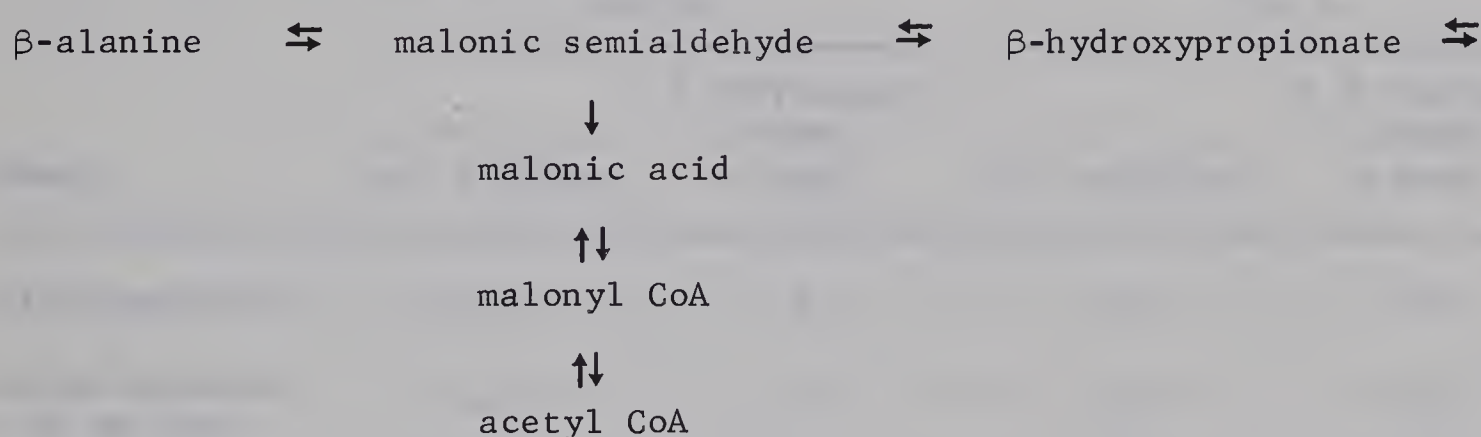
As discussed in Chapter I, other workers used a variety of tissues and were unable to stimulate ethylene evolution with β -alanine (Ku and Pratt, 1968 (tomato); Burg and Clagett, 1967 (banana)), but Wang et al. (1964, Penicillium digitatum) reported that the C-2 of β -alanine was heavily incorporated into ethylene. R.M. Knight (unpublished) found that excised leaves were able to convert β -alanine to ethylene. Amino acid analyses of the cotyledons used in these experiments indicated the presence of β -alanine, but unknown complicating factors did not allow quantitation. Fowden (1958) reported that β -alanine is widely distributed in plant tissues.

Variability in results did not allow a meaningful comparison of β -alanine and malonate as ethylene 'precursors'. It was found, however, that the presence of both compounds produced more ethylene than either alone.

3. α -ketoglutarate

Thompson and Spencer (1966) postulated that the initial reaction in the conversion of β -alanine to ethylene was a transamination of the amino acid to malonic semialdehyde (MSA). When α -KG (50 mM), β -alanine (50 mM), and cofactors, were added to a collection together, these authors found that the increase in ethylene evolution over a sample without α -KG, was 40%. From these results it was postulated that α -KG was serving as an amino acceptor for a β -alanine transaminase. Meheriuk (1965) obtained only a slight stimulation of ethylene generation when α -KG (1 mM) was added to a tomato particulate fraction that was 50 mM in β -alanine. The author found that when α -KG (50 mM) was added to a solubilized particulate enzyme solution that contained malonate (50 mM)

and β -alanine (50 mM) in the presence of cofactors, there was no change in ethylene evolution on a 0-2 h collection. When α -KG was added to separate samples that contained the soluble enzyme preparation, cofactors and either malonate or β -alanine, evolution of the volatile was inhibited in both samples, but inhibition was much greater in the reaction mixture that contained β -alanine (Table 4). The data of Table 4 concerned with malonate are in agreement with the postulation made above, that malonate is only simulating an ethylene precursor. The following scheme helps to explain this point:



α -ketoglutarate, when added to the sample that contained β -alanine, may inhibit ethylene evolution by binding to the active site of the transaminase, necessary for the conversion of β -alanine to MSA; α -KG is not utilized as an amino acceptor (Chapter V).

Concentrations of CoA as high as 0.8 mM had no effect on ethylene biosynthesis from the soluble system. Thompson (1966) reported a very slight stimulation with coenzyme A, while evolution from a particulate fraction from tomatoes, that was 50 mM in β -alanine, was inhibited by CoA (Meheriuk, 1965).

I. The Failure of Malonate to Form Malonic Semialdehyde

An experiment was set up with 1 μ c of malonate-2- 14 C to dispel or confirm the hypothesis that malonate is not a precursor of ethylene

Table 4. Effects of α -ketoglutarate on β -alanine- or malonate-stimulated ethylene evolution.

Sample	Collection period			
	0-2 h		2-4 h	
	$m\mu$ l ethylene	% difference from no α -KG	$m\mu$ l ethylene	% difference from no α -KG
50 mM malonate	16.5	0	7.0	0
50 mM malonate + 50 mM α -KG	14.0	-14	6.0	-14
50 mM β -alanine	24.2	0	29.8	0
50 mM β -alanine + 50 mM α -KG	14.6	-40	15.5	-48

Samples contained ATP (1.5 mM), TPP (2.0 mM), $MgSO_4$ (1.0 mM), pyridoxal phosphate (0.5 mM), NADH (0.83 mM) and TES (10 mM), pH 7.0. Approximately 82 mg protein were in a volume of 20 ml.

(see H above). Radioactive malonate ($3.4 \mu\text{c}/\mu\text{mole}$) was added to a collection that contained enzyme, NADH (0.83 mM), ATP (1.5 mM), TPP (2.0 mM), MgSO_4 (1.0 mM), and pyridoxal phosphate (0.5 mM). Incubation was for 2 h, and then radioactivity in malonic semialdehyde (MSA) was investigated as outlined in D.6 of Chapter II.

The R_F values of the 2,4-dinitrophenyl hydrazone of MSA in (a) benzene:acetic acid (19:1), (b) triethylamine:ether:water:pyridine (60:40:20:20), and (c) ethanol:butanol:0.5 M ammonia (70:50:15) were 0.18, 0.50, and 0.33, respectively.

No radioactivity could be detected in the silica gel scrapings that corresponded to the 2,4-dinitrophenyl hydrazone of MSA, after initial chromatography in benzene:acetic acid and rechromatography in triethylamine:ether:water:pyridine, or in ethanol:butanol:0.5 M ammonia. In addition, no radioactivity could be detected in the 2,4-dinitrophenyl hydrazone of acetaldehyde, a decomposition product of the MSA hydrazone. Very little ethylene was evolved and it was not labelled. A separate experiment that contained $50 \mu\text{c}$ of malonate-2- ^{14}C ($5 \mu\text{c}/\mu\text{mole}$), NADH (3.3 mM) and standard amounts of ATP, TPP, MgSO_4 and pyridoxal phosphate, produced $31 \text{ m}\mu\text{l}$ of ethylene but no ethylene- ^{14}C . If the ethylene did originate from malonate carbons then label should have been incorporated into the volatile. It appears that malonate is not an ethylene precursor and its function in promoting ethylene biosynthesis is one ^{of} preventing the loss of carbons from the pathway.

What compounds then form ethylene in the malonate collections? It was suggested earlier that intermediates in the pathway may be bound to enzymes and that the presence of malonate forces them through.

Proteolytic enzymes and nucleic acid degrading enzymes may provide amino acids and β -alanine (Tsai and Axelrod, 1965) to serve as ethylene precursors.

J. An Investigation of the Steps Involved in the Conversion of β -alanine to Ethylene

1. Addition of the Intermediates

Experiments carried out to compare the ability of the intermediates (β -alanine, MSA, β -hydroxypropionate, and acrylate) to stimulate ethylene biosynthesis in the soluble system were much less rewarding than the experiments with β -alanine alone (Figure 13). It was found that β -alanine resulted in a very large increase in biosynthesis but that the compounds postulated to be 'on route' from β -alanine to ethylene gave only a slight stimulation over that achieved with β -alanine. Ten determinations that contained various concentrations of the intermediates, NADH (0.83 mM), standard amounts of ATP, TPP, MgSO_4 and pyridoxal phosphate and TES (50 mM), pH 7.2, were averaged to obtain the following: β -alanine, 9.8 μl ethylene; β -hydroxypropionate, 11.2 μl ethylene; and acrylate, 10.9 μl ethylene. Four determinations were averaged to obtain the following: β -alanine, 11.0 μl ethylene; MSA, 11.7 μl ethylene; and β -hydroxypropionate, 12.6 μl ethylene. Thus, it was established that MSA could stimulate more than β -alanine and that β -hydroxypropionate could stimulate more than MSA. The small increases over β -alanine could indicate that the interconversions of β -alanine, MSA, and β -hydroxypropionate do not limit the overall scheme leading to ethylene.

Acrylate (<5 mM) stimulated more than β -alanine but less than β -hydroxypropionate. Jacobsen and Wang (1965) reported that

Penicillium digitatum converted acrylate-2(3)- ^{14}C to ethylene but postulated that fumarate was the acrylate precursor. Thompson (1966) reported that 75 mM acrylate inhibited evolution of the volatile by 70% and speculated that the extreme reactivity of the compound allows it to attack other components, such as essential sulfhydryl groups of enzymes, in the reaction mixture. Thijsse (1964) has reported that acrylate (14 mM) inhibited the β -oxidation of lipids; β -oxidation is involved in the formation of ethylene from β -alanine. If the inhibition is brought about by the interaction of the double bond of acrylate with sulfhydryl groups, then if these groups were protected, the inhibitory action of acrylate may be prohibited.

2. Acrylate Stimulation of Ethylene Evolution in the Presence of Dithiothreitol

As outlined in Chapter I, plant enzymes that link acrylate, β -hydroxypropionate, and MSA have been described. The two enzymes involved in the conversion of β -alanine to ethylene that have not previously been described in plants are (1) β -alanine transaminase and (2) acrylic decarboxylase. The transaminase is reported on in Chapter V. In order to give acrylate a firmer role as the most immediate ethylene precursor in the β -alanine system, acrylate was investigated in the presence of dithiothreitol (DTT). The use of DTT as a sulfhydryl group protector (Cleland, 1964) was discussed earlier in this chapter. The dithiol at two concentrations was added to ethylene collections that contained various levels of acrylate (sodium salt, K and K Co.) (Figure 14). The following observations were made: (1) As the concentration of DTT was increased, ethylene evolution increased. (2) The more DTT present, the greater the augmentation in ethylene

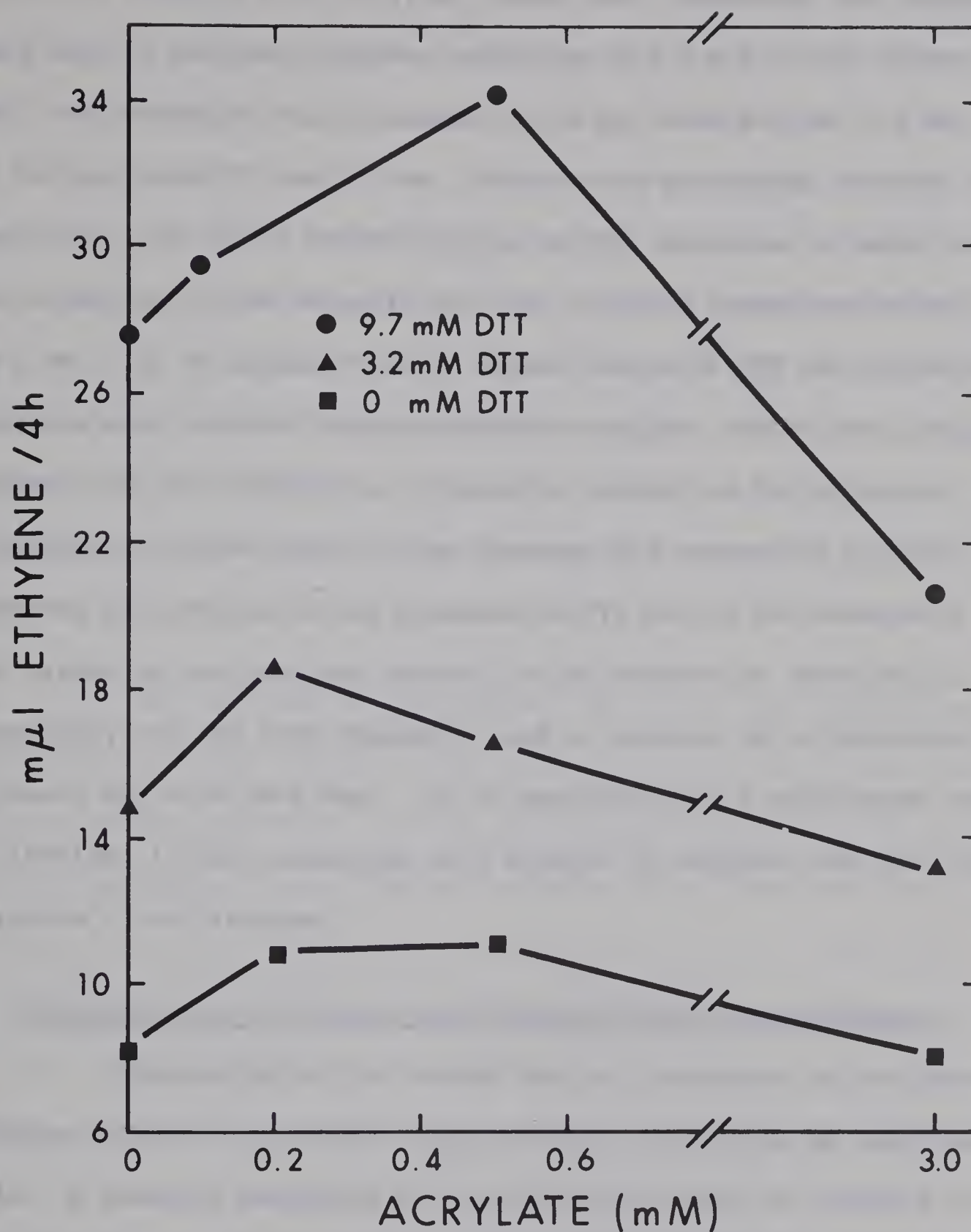


Figure 14. Acrylate stimulation, in the presence of DTT, of ethylene evolution. Reaction mixtures contained ATP (1.5 mM), TPP (2.0 mM), MgSO_4 (1.0 mM), pyridoxal phosphate (0.5 mM), and TES (50 mM), pH 7.2. Approximately 82 mg of protein were in a volume of 20 ml.

evolution obtained when acrylate levels were increased, ie: acrylate was only able to increase ethylene evolution by 1.9 μl in the absence of DTT, but production was increased by 3.8 μl when DTT was 3.2 mM, and by 6.6 μl when DTT was 9.7 mM. However the percentage increase remained constant. (3) At all concentrations of DTT, there was a large drop in the evolution of the volatile when the acrylate concentration was raised to 3 mM. (4) It appears that at higher levels of DTT the system will tolerate more acrylate before inhibition begins. These data certainly suggest that DTT exhibits a 'protective' effect on the ethylene biosynthetic system, both in the presence and absence of acrylate. No ethylene was evolved in the presence of DTT but in the absence of protein. The origin of the ethylene carbons in the absence of substrate is uncertain, but the same arguments used to explain the stimulation by malonate may also hold here. It is possible that a multienzyme system is involved in the conversion of β -alanine to ethylene and that 'free' acrylate is not involved.

3. Incorporation of Tritium from Tritiated Water into Ethylene

Examination of the scheme for the conversion of β -alanine to ethylene (Chapter I) reveals that the water medium has an important role. A possible mechanism for the decarboxylation of acrylate is given by Thompson (1966, P.64). This mechanism involves TPP and is similar to that generally accepted. A study reveals that two protons are incorporated into the ethylene molecule, one proton on each carbon. If there is an equilibrium between each step and the proton source is the medium where tritium is present, it is possible for all four 'hydrogens', of the ethylene molecule to be labelled.

Collections were set up as usual except that the protein solution was freeze-dried to reduce the final volume by one half or less, in order to increase the specific activity of the water medium. The sample in the first experiment was 50 mM in malonate, 50 mM in β -alanine, and 3.3 mM in NADH, and contained standard amounts of ATP, TPP, MgSO_4 and pyridoxal phosphate, 150 μc $^3\text{H}_2\text{O}$ (0.270 $\mu\text{c}/\mu\text{mole}$), and TES (50 mM), pH 7.2 in a final volume of 10 ml. Collections were made for 0-6 and 6-18 h. Radioactivity in the ethylene was determined as described in Chapter II and a calibration curve for the GRC is shown in Figure 15. Unfortunately, ethylene- ^3H was not available to calibrate the instrument and an efficiency one half of that obtained for ^{14}C was assumed.

The activity of the enzyme used in the first experiment was poor and although ethylene was evolved, no radioactivity was detected. In the second experiment, a more active preparation was used, and in addition, substrate and cofactor concentrations were increased by 20% because the final volume was 8 ml rather than 10 ml. Collection periods were extended (0-16 h and 16-23 h) and the specific activity of the $^3\text{H}_2\text{O}$ was increased to 0.338 $\mu\text{c}/\mu\text{mole}$. Table 5 shows the results of the second experiment. Radioactive ethylene was detected in both collection periods, and the total ethylene evolved was three times that of the first experiment. The specific activity of the evolved ethylene (Table 5) doubled on the second collection period, and thus, the ratio of the specific activities of the $^3\text{H}_2\text{O}$ and ethylene- ^3H also increased. This increased specific activity may indicate either the influence of bound intermediates or greater operation of a pathway, other than the β -hydroxypropionate route, during the first collection period. The low specific activity ratio of 0.60 (1.0 is the theoretical maximum) can be

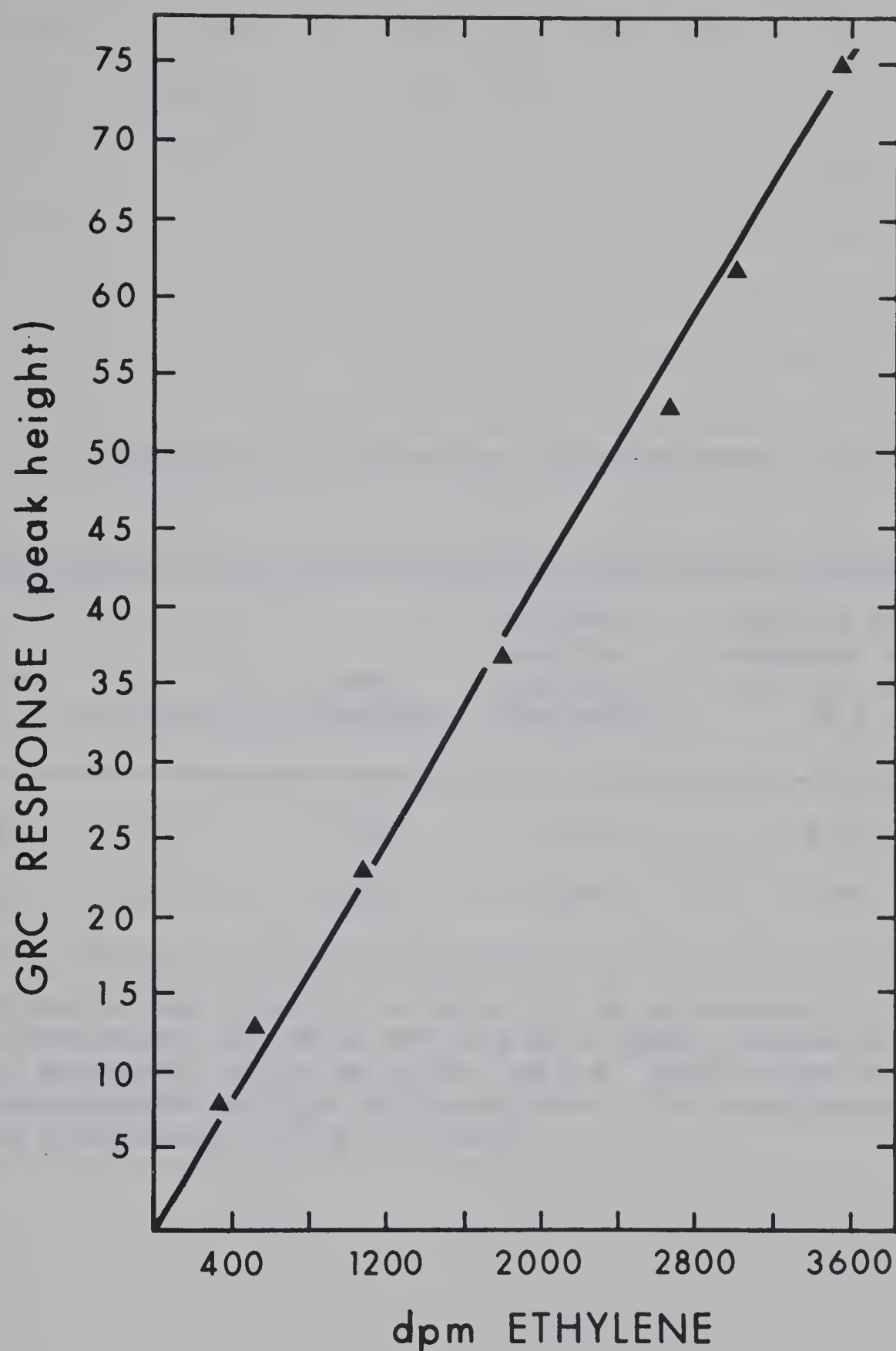


Figure 15. Calibration curve of the gas radiochromatograph (GRC). The 85 ml chamber was flushed with high purity methane (30 cc/min). Polarizing voltage was 2700 volts, the time constant was 10 sec and full scale on the recorder represented 1500 dpm.

Table 5. Incorporation of tritium from tritiated water into ethylene.

Collection period	m μ l ethylene	dpm ethylene	Ethylene specific activity (μ c/ μ mole)	Specific activity ethylene \div specific activity $^3\text{H}_2\text{O}$
0-16 h	90	970	0.110	0.33
16-23 h	25	480	0.196	0.60

Reaction mixture was 60 mM in β -alanine, 60 mM in malonate, 4.0 mM in NADH, 1.8 mM in ATP, 2.4 mM in TPP, 1.2 mM in MgSO_4 , 0.6 mM in pyridoxal phosphate, and 60 mM in TES, pH 7.2. Final volume was 8 ml and contained 150 mc $^3\text{H}_2\text{O}$ (0.338 μ c/ μ mole). The reaction mixture contained approximately 82 mg of protein.

attributed to the fact that either the enzymes that synthesize ethylene from β -alanine were not in complete equilibrium with the medium ($^3\text{H}_2\text{O}$) or that ethylene biosynthesis occurred by more than one pathway, one of which which does not involve steps that allow for the incorporation of tritium. The finding by Burg and Thimann (1959) that tritium from $^3\text{H}_2\text{O}$ was rapidly incorporated into ethylene suggested to them that one of the terminal steps in the biosynthesis may be a dehydration. This finding and subsequent postulation are in agreement with the data of Table 5, and are in accordance with the proposed scheme for the conversion of β -alanine to ethylene.

K. Ethylene Biosynthesis by Cytoplasmic Enzymes

The supernatant from the subcellular fraction pellet contains cytoplasmic enzymes. This cytoplasmic enzyme system was prepared as outlined in Chapter II (D.3), and was investigated for its ability to synthesize ethylene from β -alanine alone, and in the presence of solubilized enzymes from the particulate fraction (Table 6). The particulate fraction was by far the better source of enzymes capable of synthesizing ethylene, but the combination of the two enzyme systems resulted in greater evolution than was obtained from either alone. The data indicate that while the subcellular particulate fraction played a major role in the biosynthesis of ethylene from β -alanine, addition of cytoplasmic enzymes resulted in a 100% increase in evolution of the volatile. When the data were converted to a specific activity (corrected for amount of protein), the soluble particulate enzymes were still superior to the cytoplasmic enzymes. Perhaps an enzyme essential for the conversion of β -alanine to ethylene is limiting in the particulate system because this enzyme is localized primarily in the cytoplasm.

Table 6. Ethylene biosynthesis by cytoplasmic enzymes alone, and in the presence of a solubilized particulate fraction.

Enzyme Preparation	Sample	Collection period	
		0-4 h	0-4 h
		Total μl ethylene evolved	$10^2 \times \mu\text{l}$ ethylene /mg protein
1	Cytoplasmic enzymes	6.6	6.6
	Particulate fraction	9.9	18.7
	Cytoplasmic + particulate	20.9	13.6
2	Cytoplasmic enzymes	2.9	2.1
	Particulate fraction	3.9	8.6
	Cytoplasmic + particulate	11.5	6.4

Reaction mixture: 50 mM β -alanine, 50 mM malonate, 50 mM α -KG, 0.83 mM NADH, 1.5 mM ATP, 2.0 mM TPP, 1.0 mM MgSO_4 , 0.5 mM pyridoxal phosphate, 50 mM TES, pH 7.2. Final volume was 20 ml. Protein was determined as outlined in Chapter II.

In a separate experiment, the cytoplasmic enzyme solution was heated in a boiling water bath for 10 min before addition of the solubilized particulate enzyme solution. The heated cytoplasmic enzyme solution failed to stimulate ethylene biosynthesis in the soluble particulate enzyme system. This finding indicated that the stimulatory factor in the cytoplasmic fraction was heat labile, and therefore, possibly an enzyme.

L. Ethylene Evolution by Excised Leaves and Cotyledon Slices Fed β -alanine-2- ^{14}C

1. Excised Leaves

β -alanine-2- ^{14}C was incorporated into excised leaves as outlined for the incorporation of L-methionine- ^{14}C (U) (Chapter II). In the first experiment, the leaves incorporated 1.1 million dpm and in the second, they incorporated 5.25 million dpm of β -alanine-2- ^{14}C . The ethylene evolved for the first 4 h after incorporation was collected and analyzed. Although significant amounts of ethylene were evolved, only a trace of radioactivity was detected in only one experiment. Radioactive ethylene could be detected when the leaves were fed 31 million dpm of β -alanine-2- ^{14}C ($0.417 \mu\text{C}/\mu\text{mole}$) (R.M. Knight, unpublished). The author's experiments indicated that the β -alanine incorporated was metabolized to a certain extent because: (1) The two experiments evolved a total of 40,000 dpm of CO_2 over the 8 h collection, and (2) after collection, the leaves were extracted three times with 80% ethanol, and TLC of this extract on cellulose (benzene: acetic acid: H_2O (60:20:20)) and a scan for radioactivity on a Nuclear Chicago actigraph indicated that approximately 5% of the radioactivity was in peaks other than β -alanine. It is significant however the major part of the β -alanine metabolism occurred after the tissue had

ceased to evolve ethylene. The data may indicate that large amounts of β -alanine are required to enable the amino acid to reach the site of ethylene synthesis.

2. Cotyledon Slices

Ten g of cotyledons were cut in slices approximately 1 mm thick and placed in buffer solution (0.3 M mannitol; and 50 mM TES, pH 7.5) that contained 44 million dpm β -alanine-2- ^{14}C ($3.43 \mu\text{C}/\mu\text{mole}$). The ethylene evolved over a 12 h period (334 $\text{m}\mu\text{l}$) was analyzed by GRC and did not contain radioactivity. The β -alanine-2- ^{14}C did not appear to be extensively metabolized as only 13,800 dpm of CO_2 was evolved over a 12 h period. Vacuum infiltration for 1 min was found to increase the dpm in CO_2 , but considerably reduce the amount of ethylene evolved.

CHAPTER V

A TRANSAMINASE ISOLATED FROM BEAN COTYLEDONS THAT CONVERTS β-ALANINE TO MALONIC SEMIALDEHYDE

The conversion of β-alanine to ethylene as proposed by Thompson (1966) requires that β-alanine is first transaminated to malonic semialdehyde (MSA). An enzyme capable of this conversion, although well studied in microorganisms and animals (Chapter I), has not been satisfactorily demonstrated in plant tissue. Thus a β-alanine transaminase was investigated in a solubilized particulate fraction from bean cotyledons that had demonstrated an ability to derive ethylene from β-alanine (Chapter IV).

Chapter II outlined the construction of a standard curve for the formazan of MSA. The curve is shown in Figure 16 and is linear except at low MSA levels.

A. β-alanine Transaminase From a Particulate Fraction

1. Amino Acceptors

Figure 17 shows the time dependent patterns of MSA formation with β-alanine and pyruvate separately, and with both of these compounds. MSA was formed in reaction mixtures that contained just one of β-alanine or pyruvate, but the majority of the activity required both substrates. A sharp drop in enzyme activity combined with the lability of the product may account for the large drop in the amount of MSA present after 2½ h. The MSA formed in the presence of only β-alanine may be enough to explain the ethylene evolved from a collection that contained solubilized particulate fraction, β-alanine, and cofactors, but no amino acceptor (Figure 13).

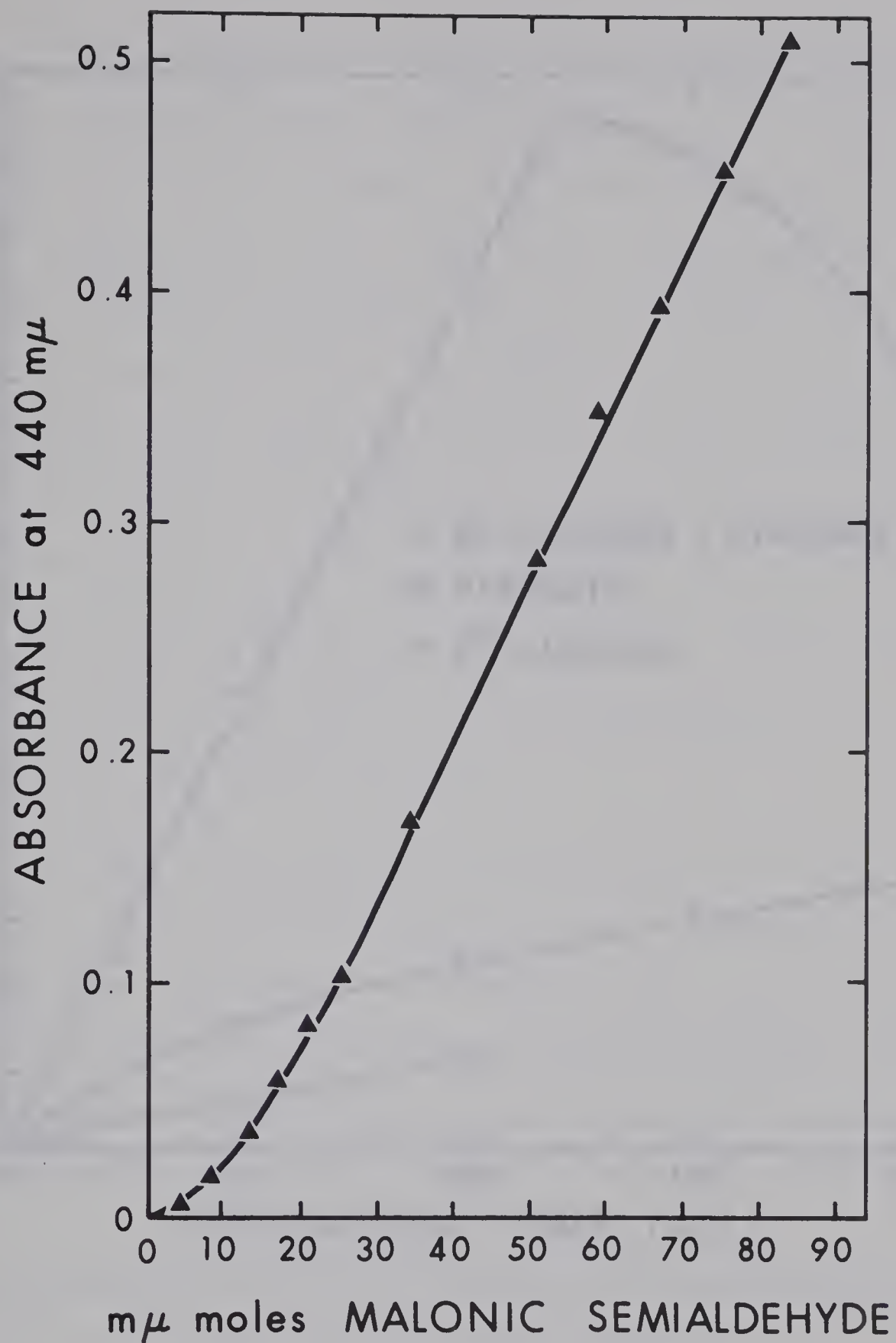


Figure 16. Standard curve for the formazan of malonic semialdehyde. Malonic semialdehyde concentration expressed as mμmoles per 1 ml original assay solution.

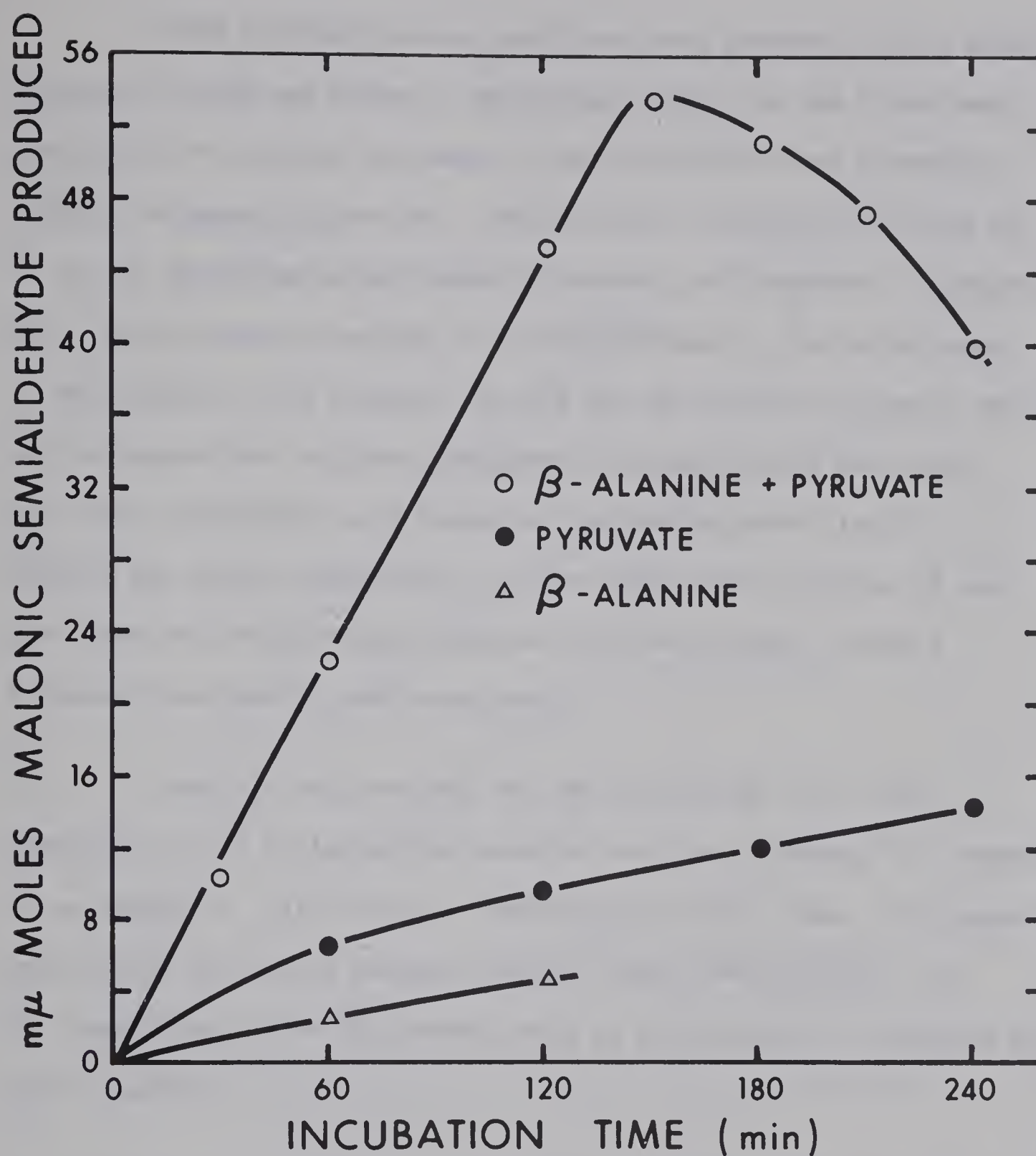


Figure 17. β -alanine transaminase activity with the indicated substrates. Each sample contained 60 μ moles of substrate, approximately 2 mg protein, and 150 μ moles of TES in a final volume of 3.0 ml (pH 7.2). Incubation was at 33 C.

When α -ketoglutarate (α -KG) replaced pyruvate as the amino acceptor, no MSA was formed. Oxalacetate (OAA), on the other hand, resulted in very great increases in MSA production over a control without β -alanine (Figure 18). Oxalacetate, like MSA, by virtue of an active methylene group, forms a formazan, and therefore interferes with the absorbancy readings of the MSA formazan. The large amount of MSA formed in the presence of only OAA may indicate either a very active enzyme that utilizes endogenous β -alanine, side reactions with other compounds, or a change in the OAA molecule itself. To support the latter explanation, it was found that solutions of OAA that were not freshly made, resulted in a more highly coloured formazan than freshly made solutions.

Possible explanations for the increasing rate of MSA formation in the β -alanine-OAA reaction are the following: (1) removal of an inhibitor, (2) substrate inhibition at early times, (3) increased activity of the enzyme perhaps through slight 'denaturation', or (4) conversion of the MSA formed early in the reaction to malonate or other products.

2. Identification of Malonic Semialdehyde as the Reaction Product

(a) With Oxaloacetic Acid as an Amino Acceptor

Enzyme, β -alanine and OAA were incubated as usual for 2 h; after this time, the digestion was heated in a boiling water bath for 15 min, cooled, deproteinized, and the 2,4-dinitrophenyl hydrazones were made (Chapter II). When MSA is heated, it will decarboxylate to acetaldehyde. TLC of the hydrazones indicated the presence of MSA in the unheated digestion, and acetaldehyde in the heated digestion (Figure 19).

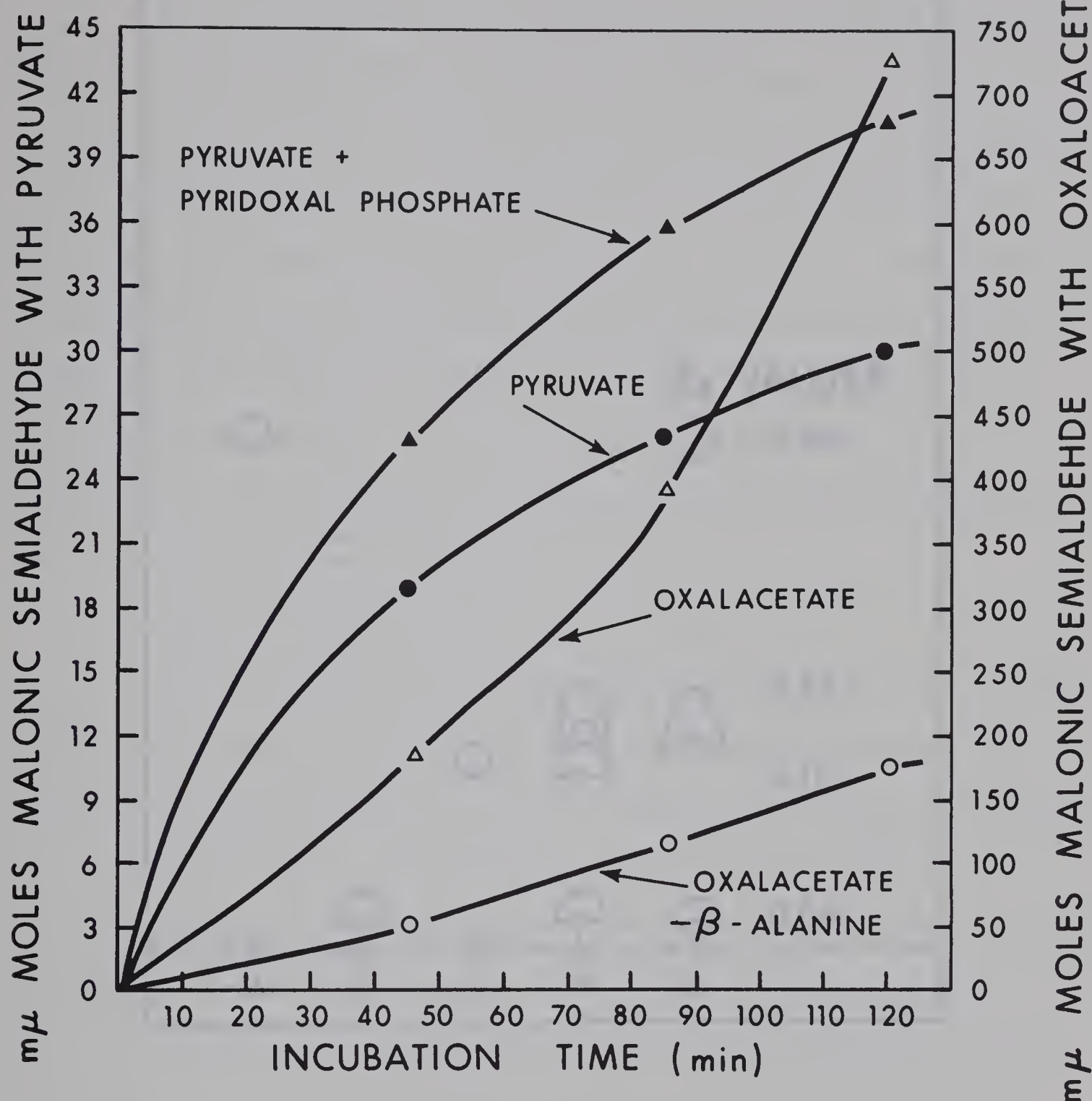


Figure 18. β -alanine transaminase activity with the indicated substrates. All reactions contained β -alanine except where indicated. Each sample contained 60 μ moles of substrate, approximately 2 mg of protein, and 150 μ moles of TES in a final volume of 3.0 ml (pH 7.4). Pyridoxal phosphate concentration was 1×10^{-4} M. Incubation was at 33 C.

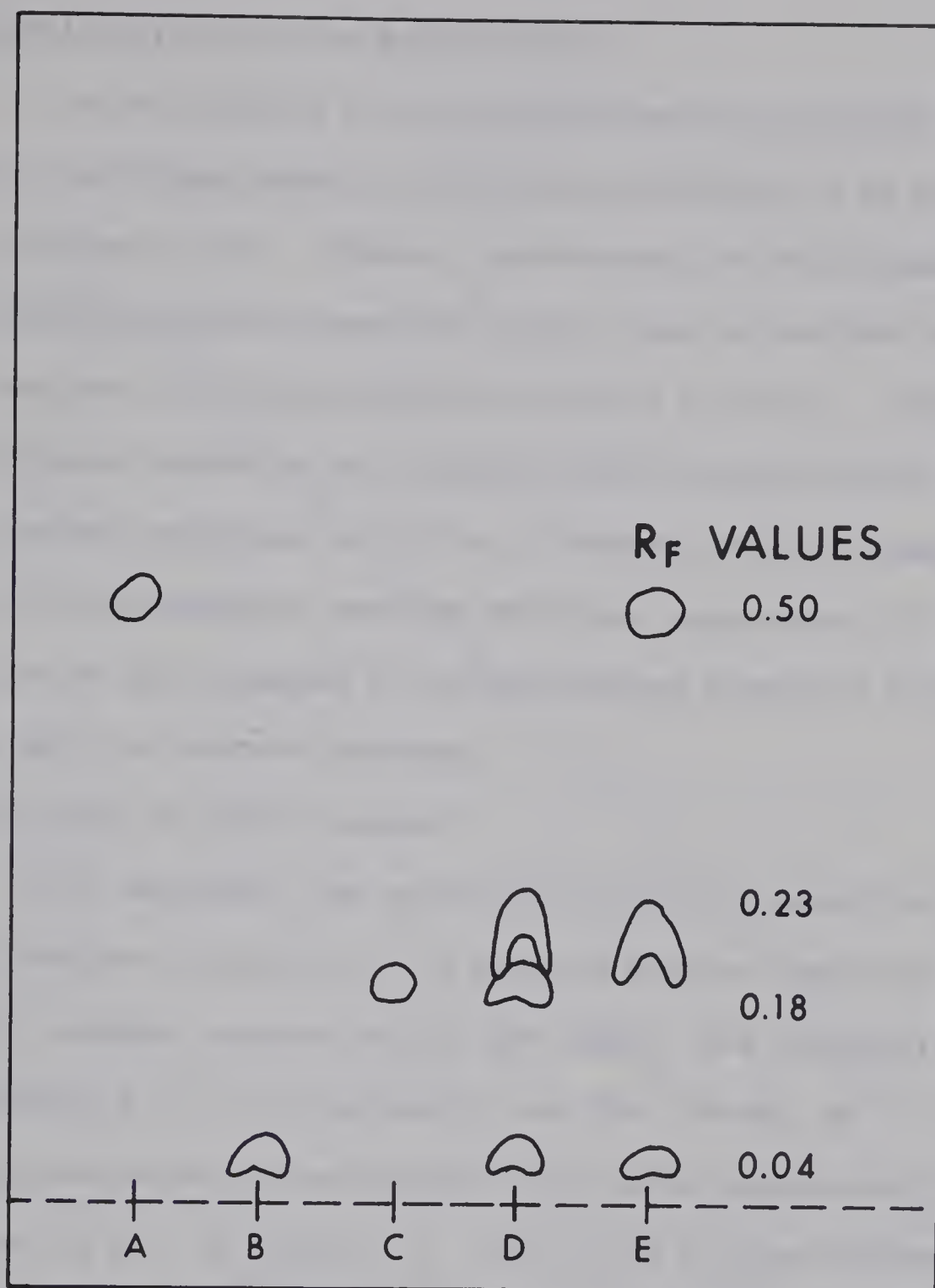


Figure 19. Silica gel thin-layer chromatogram of 2,4-dinitrophenylhydrazones from the oxaloacetate reaction mixture. Development was in benzene:acetic acid (19:1). A, B, and C are authentic samples of the hydrazones of acetaldehyde, oxaloacetate, and malonic semialdehyde, respectively. D and E represent an unheated and heated digestion, respectively. The formation of acetaldehyde in E confirms the presence of malonic semialdehyde in D.

(b) With Pyruvate as an Amino Acceptor

The low activity of the transaminase that utilized pyruvate meant that sufficient amounts of MSA were not formed to be identified as the hydrazone by TLC. However, spectroscopy of the formazan formed in the β -alanine-pyruvate reaction mixture, and of the MSA-formazan, showed that both exhibited maximum absorbance at 437 m μ . Spectroscopy of the formazan formed in the β -alanine-OAA reaction mixture did not exhibit maximum absorbance at 437 m μ . However, as the formazan was diluted, the wavelength of maximum absorbance approached 437 m μ . Interaction of the formazans of OAA and MSA may have been responsible for this shift in maximum absorbance.

(c) Without an Amino Acceptor

This experiment was carried out under the conditions normally used for ethylene collection. The reaction mixture contained NADH, (0.83 mM); standard amounts of ATP, TPP, MgSO₄, and pyridoxal phosphate; 1 μ c β -alanine-2-¹⁴C (3.43 μ c/ μ mole); and TES (50 mM), pH 7.2. Hydrazones were made, chromatographed, and their radioactivity determined as outlined in D.6. of Chapter II. TLC of the 2,4-dinitrophenyl hydrazone of MSA in (a) benzene:acetic acid (19:1), (b) triethylamine: ether:water:pyridine (60:40:20:20), and (c) ethanol:butanol:0.5 M ammonia (70:50:15) yielded these respective R_F values: 0.18, 0.50, and 0.33. A quench curve (Figure 20) was used to correct for the effects of silica gel and color on the efficiency of counting (Chapter II, D.6.). Initial chromatography showed the hydrazone of MSA to contain 2,911 dpm. However, it was found that TLC (a process shown to cause the destruction of MSA) reduced the dpm to 215. In any case, it was shown that MSA could be formed in the absence of an added amino acceptor.

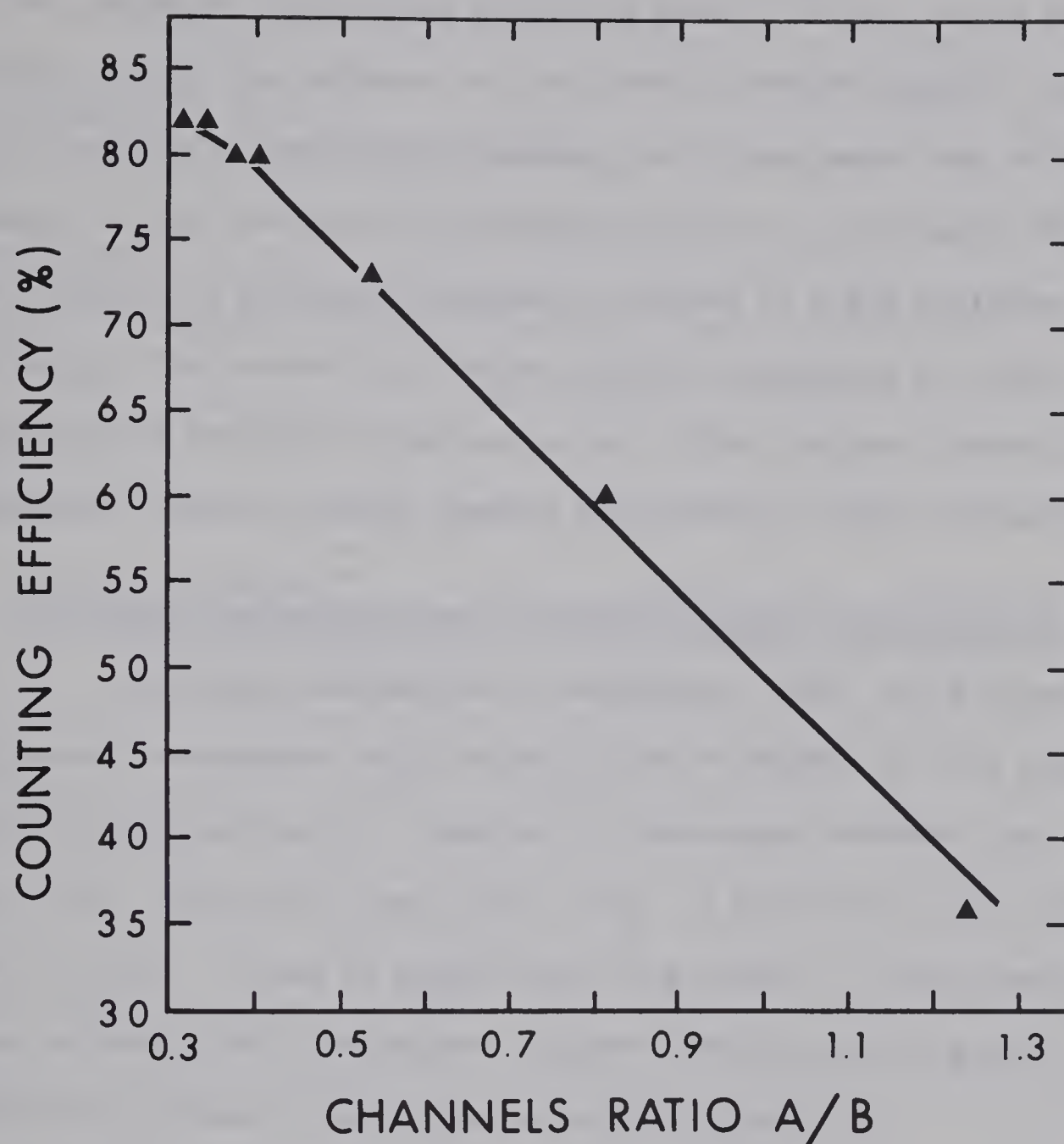


Figure 20. ^{14}C quench curve. Vials contained 110,000 dpm β -alanine-2- ^{14}C , a standard amount of silica gel, and various amounts of hydrazone (Chapter II).

3. Necessity of Pyridoxal Phosphate

The fact that a stimulation in MSA formation could be achieved by the addition of pyridoxal phosphate even after the enzyme had been incubated with the cofactor and the unbound portion removed, exemplifies the necessity of pyridoxal phosphate, and illustrates that sufficient amounts of the coenzyme for maximum activity were not bound (Figure 18). The addition of pyridoxal phosphate resulted in a 40% increase in activity. The necessity of added pyridoxal phosphate has been shown for both the bacterial (Hayaishi et al., 1961; Durham, Jacobs, and Ferguson, 1964) and animal (Baxter and Roberts, 1958) transaminases.

4. Glutamate-Oxaloacetate and Glutamate-Pyruvate Transaminases

Glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) activities were checked in this enzyme system; transaminase kits supplied by Boehringer Mannheim Corp. were utilized. GOT activity was found to be 7.5 times more active than GPT activity. It may be significant with regard to tissue metabolism that β -alanine-OAA transaminase activity was many times greater than β -alanine-pyruvate transaminase activity (Figure 18).

5. β -alanine Transaminase and Ethylene Production

None of the amino acceptors investigated in the β -alanine transaminase assay was found to stimulate ethylene biosynthesis, as pyruvate was without effect, and OAA and α -KG inhibited. It must be assumed that if the transamination of β -alanine to MSA is involved in ethylene biosynthesis, this step does not limit the reaction. It is not surprising then, that although OAA was a very effective substrate for the enzyme, this acid did not stimulate ethylene production. It

is of note that α -KG inhibited ethylene formation, and as well, failed to promote the transamination of β -alanine.

Oxaloacetic acid failed to support the β -alanine transaminases of Pseudomonas (Hayaishi et al., 1961), beef brain (Baxter and Roberts, 1958) and hog kidney (Kupiecki and Coon, 1957), but has not been investigated in a plant system. The data reported here clearly indicate that both pyruvate and OAA can serve as amino acceptors for a plant β -alanine transaminase. The two acceptors may react with two separate enzymes.

6. General Discussion

The above experiments demonstrate the presence of an enzyme that converts β -alanine to MSA, and β -alanine is thereby linked to the modified β -oxidation scheme described by Giovanelli and Stumpf (1958). The results also suggest a reason why investigators have been unable to detect a β -alanine transaminase in plant tissue; all of the assays done with plant tissue have utilized α -KG as amino acceptor, and the present author's results indicate that α -KG may not function in this role.

B. The Cytoplasmic Enzyme

A β -alanine transaminase that utilized either pyruvate or α -KG could not be detected in the cytoplasmic enzyme system, although a transaminase that utilized OAA appeared to be present. Dixon and Fowden (1961) reported that γ -aminobutyrate-pyruvate transaminase (an enzyme that is present in animal preparations, and will also transaminate β -alanine) was "concentrated in the mitochondria".

CHAPTER VI

CONVERSION OF METHIONINE TO ETHYLENE

A. By Excised Leaves

Information in Table 7 demonstrates that excised leaves are capable of converting methionine to ethylene. Confirmation that the GRC peak represented radioactive ethylene was obtained from chromatography of standard ethylene- ^{14}C (U) samples. As well, when the GC and GRC chromatograms from the L-methionine- ^{14}C (U) experiments were superimposed it was found that the leading edges of the ethylene peak (3) and the GRC (4) coincide. The 85 ml chamber of the GRC has a long 'flush out' time and the result is a broad radioactive peak (4). Peaks 2 and 5 represent impurities in the air used to sweep the chamber. The ethylene evolved in both experiments had a low specific activity, indicating that the methionine incorporated was contributing little to total ethylene evolved. When collection of the ethylene was delayed until 4 h after incorporation of the radioactive methionine (experiment (2)), the specific activity of the evolved ethylene was almost four times greater than when collection was delayed only 1 h. This indicates that the methionine incorporated may become more prominent with time, perhaps after endogenous precursors have been exhausted.

Burg and Clagett (1967) found L-methionine- ^{14}C (U) was converted to ethylene in pea stem segments (previously treated with indoleacetic acid to promote synthesis of the volatile), green banana slices, and apple sections. The specific activity of the ethylene derived from the pea stem segments was 13% of the theoretical when a

Table 7. Conversion of L-methionine- ^{14}C (U) to ethylene by excised wax bean leaves.

dpm L-meth- ionine- ¹⁴ C (U) (17.15 μc/μmole) incorporated	Collection period				Specific activity of ethylene col- lected over 4 h (μc/μmole)
	0-2 h		2-4 h		
	mμl ethylene	dpm ethylene	mμl ethylene	dpm ethylene	
4.73 x 10 ⁶ (1)	234	2,340	194	1,580	0.093
23.10 x 10 ⁶ (2)	92	3,440	109	2,860	0.320

Ethylene collections were begun 1 h and 4 h after the leaves were first put in the radioactive methionine solutions in experiments (1) and (2), respectively. The leaves used in experiment (1) were 16 days old and the leaves used in experiment (2) were 19 days old (Chapter II). Radioactive analyses were done by GRC.

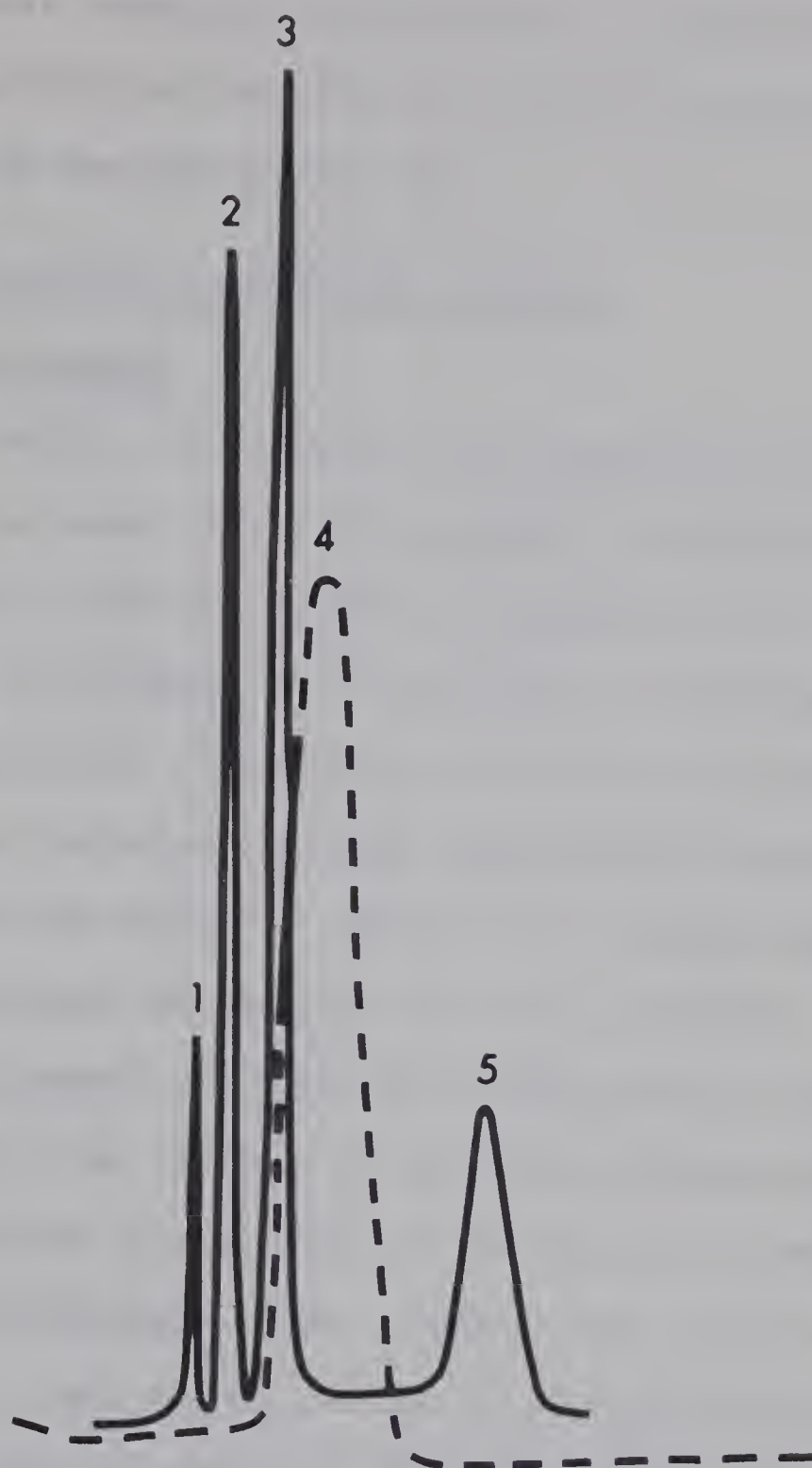


Figure 21. Superimposed tracings of the gas chromatograph (GC) and the gas radiochromatograph (GRC) recordings of an ethylene sample collected from excised bean leaves fed 23.1 dpm L-methionine- ^{14}C (U). Solid tracing is the GC response, and the hatched line is the GRC response. The baseline of the latter was purposely lowered. Peaks are as follows: 1, air; 2, unknown; 3, ethylene; 4, ethylene- ^{14}C ; and 5, unknown.

mole for mole conversion of methionine to ethylene was assumed. The specific activity of the ethylene derived from experiment (2) (Table 7) was 5% of the theoretical value.

B. By a Solubilized Particulate Fraction

1. General Studies

Studies concerned with the conversion of β -alanine to ethylene by a soluble enzyme system were reported in Chapter IV. Methionine was also found to stimulate ethylene biosynthesis in this system. A comparison of β -alanine and L-methionine as 'precursors' of the volatile appears in Table 8. The increase in ethylene over control, when L-methionine was added was three times greater than the increase caused by β -alanine and malonate. Table 8 also indicates that the stimulation by methionine was 70% inhibited by a heat treatment. The β -alanine conversion (Chapter IV) was almost 100% inhibited; this may indicate that a part of the ethylene derived from methionine may be nonenzymic. A 37% inhibition of production of the volatile by addition of diethyldithiocarbamate (1 mM), suggests that a metal enzyme (Hochster and Quastel, 1963) may be involved in the conversion of methionine to ethylene. Mapson and Wardale (1967) investigated ethylene evolution derived from methional by various enzyme fractions prepared from cauliflower florets, and these authors found a marked inhibition of ethylene production by copper and iron chelators. Apart from the work presented in this dissertation, the system of Mapson and Wardale (1967) is the only cell-free system capable of a conversion of methionine to ethylene, but the latter system requires 20 h of incubation before any stimulation can be obtained; no mention has been made of a check on bacterial contamination.

Table 8. A comparison of β -alanine and L-methionine as ethylene precursors, and the effect of heating and diethyldithiocarbamate on ethylene biosynthesis from L-methionine.

	Four h ethylene collection	
	m μ l	% of control
No added substrate	13.6	0
5 mM β -alanine + 5 mM malonate	18.0	+32
5 mM L-methionine	27.3	+100
5 mM L-methionine	35.2	0
5 mM L-methionine (protein heated)	10.4	-70
5 mM L-methionine + 1 mM diethyldithio- carbamate	22.1	-37

Each collection was 0.83 mM in NADH, 1.5 mM in ATP, 2.0 mM in TPP, 1.0 mM in MgSO_4 , 0.5 mM in pyridoxal phosphate, and 50 mM in TES (pH 7.2). Each sample contained approximately 82 mg of protein in a final volume of 20 ml. When the enzyme solution was heated it was held in a boiling water bath for 10 min, cooled, and cofactors and methionine added. When diethyldithiocarbamate was used it was incubated for 10 min at 25 C with the enzyme before methionine and cofactors were added. The substrate comparison and inhibition experiments were done on two different enzyme preparations.

Yang et al. (1967) reported that methionine could be converted to ethylene in the presence of flavin mononucleotide and light, and Patton (1954) and Enns and Burgess (1965) reported a similar mechanism for the conversion of methionine to methional. The present author's collections were made under a light intensity of 140 ft-c, but the 'extensive dialysis' to which the enzyme extract was subjected should have removed compounds such as riboflavin that catalyze the nonenzymic conversion of methionine to ethylene. In any case, this author found that methionine was able to stimulate ethylene production even in the dark.

To obtain a significant stimulation of ethylene from the solubilized particulate fraction, the present author had to add a fairly high concentration of methionine (0.5 mM gave little ethylene). Thus, in order to obtain labelled ethylene, large amounts of radioactive methionine were necessary; a reaction mixture that was 0.5 mM in methionine, with 10 μ c of L-methionine-¹⁴C (U) gave only a trace of radioactive ethylene.

2. Methional as an Intermediate in the Conversion of Methionine to Ethylene

(a) Ethylene from Methional

Ethylene production from methional has been reported to be much greater than production from methionine, both in model systems (Lieberman et al., 1965) and in natural systems (Mapson and Wardale, 1967). The author had a similar finding. However, further examination of the methional system revealed that it was impossible to separate any enzyme-mediated ethylene production from the very large amount of the volatile produced nonenzymically. Table 9 summarizes these

Table 9. Ethylene production from methional in the presence and absence of enzyme and cofactors.

	m μ l ethylene produced	
	0-2 h	2-4 h
1 mM methional alone	2	4
1 mM methional + cofactors	1,630	8,150
1 mM methional + cofactors + 31 mg enzyme solution	202	885
1 mM methional + cofactors + 31 mg heated enzyme	3,260	2,080
1 mM methional + 51 mg enzyme solution	82	88
1 mM methional + 51 mg heated enzyme solution	180	not determined

Gas chromatography of the methional on a 3 ft glass column packed with Chromosorb P and 10% Carbowax 20 M gave only 1 peak. Cofactors used were: NADH, 0.83 mM; ATP, 1.5 mM; TPP, 2.0 mM; MgSO₄, 1.0 mM; pyridoxal phosphate 0.5 mM; and 50 mM TES, pH 7.2. When the enzyme solution was heated it was held in a boiling water bath for 10 min, cooled, and methional and cofactors added.

investigations. The large amount of ethylene (collected over a 4 h period) derived from methional in the absence of protein, was reduced 88% by the addition of enzyme solution. This inhibition was partially alleviated when the enzyme solution was given a previous heat treatment. The omission of cofactors from the reaction did not help to discern if any of the gas was enzymically produced. The use of a heated enzyme solution again increased production of the gas over that with the unheated enzyme solution. A closer examination of the ethylene derived from methional in the presence of the individual cofactors showed that pyridoxal phosphate was by far the best stimulant. When all cofactors were added together there was a very large synergistic increase in ethylene evolution. It seems reasonable to conclude that a major portion of the ethylene produced from methional in the presence of enzyme extract was nonenzymic, and it was impossible to distinguish if even a small fraction originated from an enzymic conversion.

(b) Methional from Methionine

Collections that contained enzyme extract, cofactors, and 5 μ c of L-methionine- ^{14}C (U) (17.15 μ c/ μ mole) and analyzed as outlined in Chapter II (E.2) were found to contain methional- ^{14}C (Table 10). The efficiency of scintillation counting was adjusted to 100% by the use of a quench curve (Figure 20, Chapter V). TLC of the 2,4-dinitrophenyl hydrazone of methional in (a) benzene:acetic acid (19:1), (b) water saturated benzene, and (c) benzene:petroleum ether (bp 30-60 C) (3:1) yielded these respective R_F values: 0.46, 0.37 (run twice), and 0.24 (run twice). In a separate experiment two collections were set up, one contained the normal protein (45 mg) and the other contained heat denatured protein. Both contained 2.5 μ c L-methionine- ^{14}C (U) (17.15

Table 10. Radioactivity in the 2,4-dinitrophenyl hydrazone of methional, purified by thin-layer chromatography in various solvent systems.

Solvent combinations	Disintegrations per minute	
	0-2 h	2-4 h
(a)	6,250	4,205
(b)	5,550	3,040
(c) and (a)	2,756	-
(c) and (b)	2,111	-
(c) and (c)	2,362	-
(b) and (a)	-	1,825
(a) and (a)	-	1,694
(b) and (b)	-	1,875

Solvents are as follows: (a) benzene:acetic acid (19:1), (b) water saturated benzene, and (c) benzene:petroleum ether (bp 30-60 C) (3:1); chromatograms were run twice in the latter 2 systems. Where 2 solvents are given, the hydrazone was extracted from the chromatogram run in the first solvent, and rechromatographed in the second solvent (-, not determined).

The reaction mixtures from which the hydrazones were made were as follows: 1.5 mM ATP, 2.0 mM TPP, 1.0 mM MgSO_4 , 0.5 mM pyridoxal phosphate, 0.83 mM NADH, 50 mM TES, 5 μC L-methionine- ^{14}C (U), and approximately 82 mg of protein. Final volume was 20 ml, pH 7.2.

$\mu\text{C}/\mu\text{mole}$) and standard cofactors (Table 9). After a 3 h incubation in the dark, hydrazones were made and chromatographed twice in water saturated benzene. The 2,4-dinitrophenyl hydrazone of methional from the heat denatured sample contained 457 dpm whereas the unheated sample contained 3,295 dpm in the hydrazone of methional. It is apparent from the data of Table 10 that methionine can give rise to methional, and since seven times more radioactive methional was obtained from an undenatured over a denatured sample, it is probable that the conversion is enzymic.

Lieberman et al. (1965) have not been able to trap and identify methional in their model system, nor have Mapson and Wardale (1967) in their enzymic system, even though both groups postulated that methional is an intermediate in the conversion of methionine to ethylene. It is significant then, that an enzyme extract capable of cleaving methionine to methional, is also capable of mediating a conversion of methionine to ethylene. Since it has also been shown that methional can very readily form ethylene, it may be that the ethylene derived from methionine, is formed from the nonenzymic breakdown of methional, the latter having originated enzymically from methionine.

The transformation of methionine to methional may well involve the α -keto acid of methionine (α -keto- γ -methylthiobutyrate). Recently reported experiments (Chapter I) have now suggested that this compound could be a natural precursor of ethylene. It has been speculated (Yang, 1967) that methionine is converted to methional by a transaminase, and a decarboxylase, and then methional is cleaved by a peroxidase to ethylene (presumably α -keto- γ -methylthiobutyrate would be an intermediate).

Mazelis and Ingraham (1962) reported on the oxidative decarboxylation of

methionine by peroxidase. The major product of the reaction was 3-methylthiopropionamide (amide of the acid equivalent of methional), but small amounts of the α -keto acid of methionine as well as the acid equivalent of methional were also found. In consideration of these findings, it is interesting that Yang (1967) found that horseradish peroxidase cleaved methional but not methionine to ethylene.

C. By a Cytoplasmic Enzyme System

A cytoplasmic enzyme system was prepared as outlined in Chapter II (D), and a comparison of the abilities of β -alanine and L-methionine to stimulate ethylene biosynthesis in the presence of this extract was made. As with the subcellular enzyme system, methionine resulted in a greater stimulation in the evolution of the volatile than did β -alanine. The rate of evolution from the cytoplasmic system was less than 1/6 that of the particulate enzyme system.

CHAPTER VII

MISCELLANEOUS COMPOUNDS AS ETHYLENE PRECURSORS

A. Propionate

It was speculated by Thompson and Spencer (1967) that the conversion of methionine to ethylene takes place through propionate. They used this scheme to link methionine with their proposed pathway for the conversion of β -alanine to ethylene (Chapter I). If propionate is an intermediate in the conversion of methionine to ethylene, then propionate should be a better precursor than the sulfide.

Collections were set up that contained standard amounts of protein, ATP, TPP, MgSO_4 , pyridoxal phosphate and NADH, and methionine and propionate were evaluated as precursors. An average of four determinations showed that propionate addition (5 mM) resulted in 74% less ethylene than methionine addition (5 mM), and further experiments indicated that a portion of the remaining 26% may have had a nonenzymic origin, as heat denatured protein evolved more ethylene than normal protein. Meheriuk (1965) reported a 100% stimulation of ethylene evolution from a tomato particulate fraction when the assay was made 50 mM in propionate. It is interesting that Lieberman and Kunishi (1968) have reported a 40 to 50 percent increase in ethylene evolution when propionate (1 mM) was incubated with tomato fruit slices, but subsequently found that this ethylene was not derived from propionate. Instead, the propionate stimulated (by 30 percent) the incorporation of L-methionine- ^{14}C (U) into ethylene.

B. Propanal

Lieberman and Kunishi (1967) reported that 1 mM propanal stimulated ethylene production from mature green tomato slices 20% more than did methionine, and thereby postulated propanal as a precursor in this tissue. The present author found that 5 mM propanal, in the presence of cofactors and the particulate enzyme extract, generated 249 μ l of ethylene, but when the enzyme extract was replaced by a heat denatured enzyme, 395 μ l of ethylene were evolved. This finding indicated that a large amount of the ethylene evolved might have been because of a nonenzymic cleavage of propanal.

CHAPTER VIII

GENERAL DISCUSSION AND CONCLUSIONS

It has just been within the last year or so that studies on precursors for the biosynthesis of ethylene have progressed to a stage where cell-free, and even soluble systems have been developed. Unfortunately, no one precursor has been able to provide the complete answer.

A number of reports have appeared that demonstrate the ability of subcellular particulate fractions from a variety of tissues (plants, animals, and microorganisms) to produce ethylene. The author was able to show that during germination the patterns of ethylene evolution from whole bean cotyledons and from a subcellular particulate fraction isolated from these cotyledons were very similar. In addition, it was shown that the combination of a cytoplasmic enzyme system, that did not evolve ethylene itself, with an enzyme system from a particulate fraction, resulted in a considerable stimulation in ethylene production over either system alone. When these two facts are coupled with the observation that ethylene evolution from both whole cotyledons and a particulate fraction very nearly follows mitochondrial respiration (succinate as substrate) and mitochondrial ATPase activity, there is enough evidence to suggest that the particulate components of the cell, or more specifically the mitochondria, are the major area for ethylene biosynthesis in bean cotyledons.

Apart from the system studied in this dissertation, Mapson and Wardale (1967 and 1968) have reported the only other soluble system of

biological origin able to synthesize ethylene from added substrates. They found that a soluble enzyme extract from cauliflower florets was able to cleave methional with the resultant formation of ethylene. Thompson (1966) used an enzyme powder prepared from a subcellular particulate fraction of bean cotyledons, and studied the conversion of β -alanine to ethylene. Using a soluble enzyme system from bean cotyledons, the present author investigated several compounds (that have been shown to be ethylene precursors) to evaluate their ability to generate ethylene. Relative to most of the systems (mainly subcellular) reported in the literature, this soluble system was not an active one. However, even small amounts of ethylene are physiologically significant, and the evaluation of precursors should not be adversely affected by use of a system with a low conversion rate.

The results reported in this dissertation, when coupled with those of Thompson (1966) leave little doubt that bean cotyledons contain enzymes capable of generating ethylene from β -alanine, although a role for β -alanine as an ethylene precursor in certain tissues has been questioned (Chapter I). During the course of the investigation that led to this conclusion, an enzyme that converted β -alanine to malonic semialdehyde (β -alanine transaminase) was found to be present in that portion of a subcellular particulate fraction that could be rendered soluble by Triton X-100. A unsatisfying qualitative experiment is the only other report of such an enzyme in plant tissue (Tsai and Axelrod, 1965). The results reported in this thesis confirm the negative results of other workers, in that α -ketoglutarate would not accept the amino group from β -alanine. However, enzymes were found that transferred the amino group of β -alanine to pyruvate or oxalacetate.

A comparison was made of the effectiveness of β -alanine and methionine in stimulating ethylene evolution by the soluble enzyme system from bean cotyledons. The increase in ethylene evolved (over a sample with no substrate added) in the presence of L-methionine was three times greater than the increase in the presence of β -alanine. This finding may explain why Burg and Clagett (1967) were able to detect an increase in evolution of the volatile when L-methionine, but not β -alanine, was added to apple or green banana slices. However, other factors may also enter, such as supply of natural substrate, and difficulties in penetration to the site of ethylene biosynthesis.

Researchers have obtained ethylene derived from methionine and methional, and it has been postulated that methional is an intermediate in the conversion of methionine to ethylene, although all the evidence supporting this is circumstantial. The author was able to show that an enzyme system capable of catalyzing the conversion of methionine to ethylene was also capable of forming methional from methionine. The ease with which the author found methional could be nonenzymically cleaved to ethylene, suggests that the final step in the formation of ethylene from methionine may be a nonenzymic cleavage of methional. There is still the possibility of an enzymic conversion as three enzyme systems have been reported that will cleave methional to ethylene.

A number of nonenzymic model systems have been discovered that will convert a variety of compounds to ethylene. The author found that methional, propanal and propionate, compounds that have been proposed as natural precursors, were cleaved to ethylene by a mechanism

that in some instances appeared to be nonenzymic. The ease with which so many compounds form ethylene in model systems should serve to make one wary of the usefulness of model systems in the evaluation of natural precursors of ethylene.

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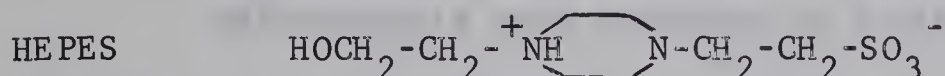
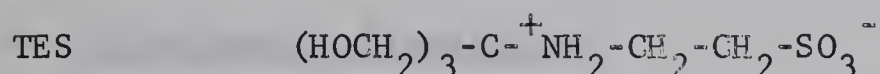
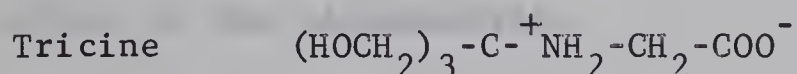
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APPENDIX

AN EVALUATION OF THE EFFECTS OF FIVE BUFFERS ON RESPIRATORY PARAMETERS OF ISOLATED MITOCHONDRIA

A. Introduction

When the author commenced the research reported in Chapter III concerned with mitochondrial respiratory parameters of bean cotyledons, he soon found Tris buffer to be inadequate for quantitative measurement of phosphorylative efficiency and respiratory control (RC). He therefore embarked on an evaluation of the two routinely used buffers, phosphate and Tris, and three little known buffers, Tricine, TES, and HEPES. The structural formulae for the latter three buffers are given below:



B. Literature Review

Several compounds have recently been proposed as suitable buffers for biochemical work in the pH range 6-8 (Good et al., 1966; Lewis, 1966; and Good, 1962). Until the introduction of Tricine by Good (1962), and more recently a number of other buffers proposed for biological work by Good et al. (1966), the selection of a buffer was generally based on the pKa value of the buffer rather than on the compatibility of the compound with the biological system under study. In 1962 Good demonstrated the superiority of Tricine, as a buffer, over Tris in the Hill reaction coupled to photophosphorylation in isolated chloroplasts. Recent work of Good et al. (1966) showed TES

and HEPES to be superior to all other buffers tested, in which to measure the respiration rate of bean hypocotyl mitochondria and cell-free bacterial protein synthesis. Bucke, Baldry, and Walker (1967) have shown that TES is superior to HEPES and Tricine for measurement of carbon dioxide fixation by isolated chloroplasts.

C. Methods

1. Growth of Seedlings

Seedlings were grown as described in Chapter II and the cotyledons were picked after 9 days. They were washed in cold demineralized water, cooled to 4 C, and stored 12 h before use. This length of storage made the experiments manageable and had no measureable effect on the mitochondria.

2. Mitochondria Preparation

Mitochondria were prepared as outlined in Chapter II, except that one of either phosphate, Tris, Tricine, TES, or HEPES replaced TES as buffer in the grind (pH 7.4) wash (pH 7.2) and assay media (pH 7.2). Tests were carried out immediately on a portion of the mitochondrial suspension, and the remainder was aged 2 h in the dark at 0 C, and the tests repeated.

3. Response to Cytochrome c

Cytochrome c (0.002 μ moles in 20 μ l) was added after the mitochondria, isolated in one of five buffers, began respiring at a constant rate. The degree of stimulation was calculated from the slopes of the tracings before and after addition of the cytochrome c.

4. Respiration Rate

Oxygen consumption, respiration rate, and determination of mitochondrial protein were as outlined in Chapter II. Respiration rate

was measured concomitant with the determination of RC and ADP/O values on mitochondria that had been isolated in one of either phosphate, Tris, Tricine, TES, or HEPES.

5. Respiratory Control and ADP/O Ratios

RC and ADP/O ratios were calculated as outlined in Chapter II, except that an amount of ADP ($0.150\ \mu\text{moles}$ per addition) was added such that at least three control values could be determined before oxygen exhaustion. ADP solutions (pH 7.2) were prepared with one of the five different assay media.

6. pKa Values of the Buffers Alone and in the Presence of the Other Components of the Assay Medium

pKa values of the buffers alone were determined by titration of 5.00 ml of a 0.05 M solution of the buffer at 24 C to the equivalence point with a Radiometer model SBR2c Titrigraph. The pKa values of the buffers in the presence of the components of the assay medium were determined from the titration curve. All buffers were titrated with certified grade 0.50 N NaOH, except Tris, which was used as the free base and titrated with 0.50 N HCl.

7. Synthesis of TES

The synthesis of TES, which was not commercially available when this work was begun, was a modification of the method of Good et al. (1966). One of the starting compounds, sodium 2-bromoethanesulfonate, was prepared by the method of Marvel and Sparberg (1943). To assure proper crystallization of the TES, it was found to be essential to recrystallize the sodium bromoethanesulfonate before its condensation with Tris. Following the 3-4 h condensation reaction, the mixture was

twice passed through a 2 X 15 in column of Bio-Rad AG 50W X 12, 50-100 mesh sulfonic acid resin in the hydrogen form. The column was regenerated with 6 N HCl, washed free of chloride, and the mixture again twice passed through the resin bed. The eluate was evaporated down to a thick syrupy liquid under vacuum on a Büchner rotary evaporator with a 70 C water bath. The product crystallized out on addition of approximately 85% ethanol (v/v). Recrystallization was best achieved by suspension of TES in boiling 95% ethanol, followed by slow addition of boiling water until the majority of the product dissolved. After filtration while hot, the solution was cooled and the crystals were collected by filtration on a Büchner funnel (m.p. of product on 4X recrystallized from ethanol-water, 223 C). Nuclear magnetic resonance analysis on the resultant crystals showed protons in $-(CH_2)_3$ at 6.13 to be equivalent, and indicated the four protons of $-CH_2-CH_2-$ in a quartet. Further corroboration of the identity of TES was provided by titration data and IR spectra. TES from a commercial source (Calbiochem) was used for comparison in most experiments.

D. Results

All experiments were carried out on three separate mitochondrial preparations and the same relative results were obtained.

1. Mitochondria Preparation

BSA has been reported to counteract the deleterious effects of Tris (Good et al., 1966; and Childress and Sacktor, 1966) and may well do the same for certain of the other buffers used, and therefore its concentration was kept at 0.1% in both the grind and wash media.

Tris and Tricine, with pK values over 8 (Table A-5) would not be expected to buffer well at pH 7.4 in a solution with a low concentration of buffer; therefore buffer concentration was made relatively high, 0.05 M, so that pH changes alone did not play too great a role in the measurements. A disadvantage peculiar to Tricine was that after the grind and wash media had been freshly made and adjusted to the proper pH at 0 C, the pH would drop 0.2 units over a 2 day period; this drop did not occur with 0.05 M Tricine alone.

The yield of mitochondria, as determined by the Lowry (Lowry et al., 1951) protein method on a portion of the final mitochondrial suspension, was found to depend on the buffer used for their isolation; in general, phosphate resulted in the greatest yields of mitochondria, followed by TES and HEPES, and then by Tricine and Tris. Tris consistently gave very poor yields and many times insufficient amounts of mitochondria were obtained to carry out all tests. This variation in yield may indicate degrees of mitochondrial clumping caused by the different buffers.

To keep the number of additions to the reaction cell to a minimum, both succinate and phosphate were included in the standard assay medium. Variation in the yield of mitochondria with the different buffers did not permit addition of a definite volume of mitochondrial suspension, but rather, mitochondria were added to obtain an arbitrary visual turbidity.

2. Respiratory Response to Added Cytochrome c

Cytochrome c is well known to be easily lost from mitochondria. For example, simple isotonic saline washing removes a large amount of

cytochrome c from rat liver (Estabrook, 1958) and flight muscle (Estabrook and Sacktor, 1958) mitochondria. Table A-1 shows typical results obtained on addition of 0.002 μ moles of cytochrome c to respiring mitochondria. The least response was elicited from mitochondria prepared in HEPES, the most from those in Tris and Tricine, while phosphate and TES were intermediate. The low response in HEPES buffer is taken to mean a low amount of cytochrome c leakage from the mitochondrion, while the reverse would be true for Tris and Tricine. Little difference was found between Tris and Tricine, both of which showed a marked response to added cytochrome c. Aging of a portion of the mitochondrial suspension in the dark at 0 C for 2 h did not change the degree of stimulation, and since indeterminate amounts of mitochondria were used, it is suggested that the response was independent of mitochondrial concentration. The aging process may cause additional leakage of cytochrome c but it would not be lost since no further washings were done.

Phosphate is known to cause mitochondrial swelling (Azzone and Azzi, 1965), and yet mitochondria that were isolated and assayed in 0.05 M phosphate buffer showed less response to cytochrome c than those treated in any other buffer except HEPES. Tris has also been reported to cause mitochondrial swelling (Childress and Sacktor, 1966). If the swelling effect of Tris is responsible for the large response to added cytochrome c, then phosphate and Tris buffers may act differently, and more than a simple swelling effect may be involved in the loss of cytochrome c.

3. Rate of Respiration

For the examination of the effects of other factors on respiration, loss of cytochrome c from the mitochondria was counteracted

Table A-1. Effect of exogenous cytochrome c on the respiration of isolated bean cotyledon mitochondria.

Buffer	Rate of oxygen utilization ($\mu\text{moles/min}$)		Percent increase in respiration after addition of cytochrome c
	Before addition of cytochrome c	After addition of cytochrome c	
phosphate	25.0	28.8	15
Tris	30.0	58.3	28
Tricine	16.7	21.7	30
TES	51.7	61.7	19
HEPES	58.3	65.0	11

Cytochrome c, 0.002 μmoles was added in 20 μl of assay medium. Assay medium composition: 0.3 M mannitol, 8 mM succinate, 4 mM MgCl_2 , 2.5 mM KH_2PO_4 , 50 mM buffer, pH 7.2. Final volume was 3.2 ml.

by its addition in a standard amount ($0.002 \mu\text{moles}/3.2 \text{ ml}$ of assay medium). Likewise, to eliminate ADP as a factor affecting respiration rates, an amount ($1.50 \mu\text{moles}/3.2 \text{ assay medium}$) was added sufficient to keep the mitochondria constantly in state 3. Distinct differences in oxygen utilization rates were found with the various buffers (Figure A-1). Mitochondria in Tris consistently gave the slowest rates while those in TES resulted in the fastest rates. Although the parameters did not change greatly in 2 h, this time was chosen as one that might normally occur in working with mitochondria. Because of the difficulty in obtaining enough mitochondria with Tris buffer to enable all of the parameters to be measured on both fresh and aged mitochondria, the results of two separate experiments were combined. With one mitochondrial preparation, all the mitochondria were used to obtain initial respiratory rates, and with a second preparation all the mitochondria were aged; the respiratory rates were related through the values obtained with phosphate buffer. The decrease in respiration rate when mitochondria were aged in HEPES-buffered medium was greater than the decreases obtained with TES, Tricine and phosphate buffers on aging; only mitochondria aged in Tris showed a larger decrease in respiratory rate.

4. Respiratory Control and ADP/O Values

In order to obtain RC values and ADP/O ratios there must be a sharp break in oxygen consumption each time the added ADP becomes exhausted, that is, the transition from state 3 (phosphate, oxygen, substrate and ADP in excess) to state 4 (ADP limiting) must be immediate. If this does not occur, then the point where phosphorylation stops cannot be accurately ascertained. A truly meaningful RC value also

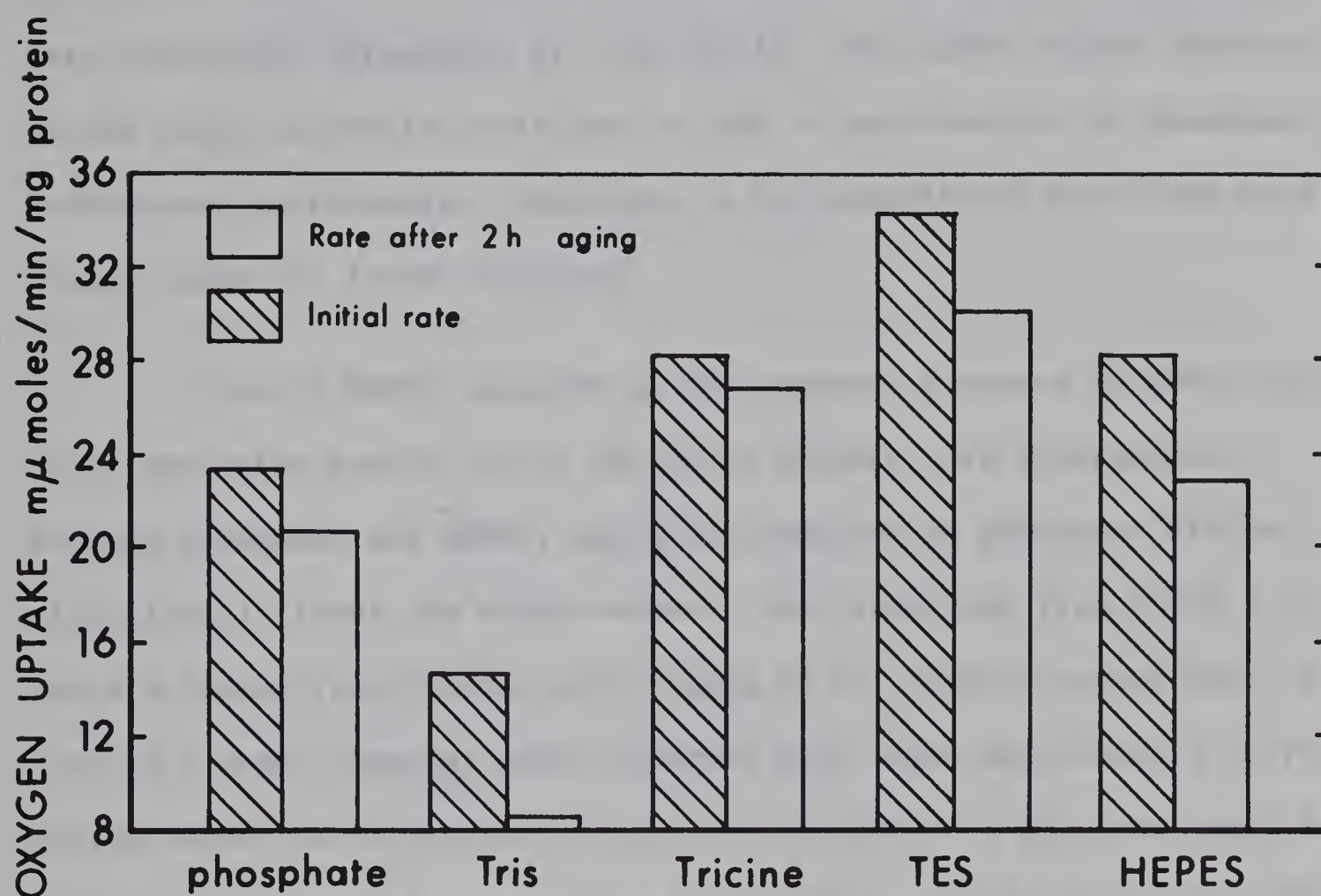


Figure A-1. Rates of respiration initially, and after a 2 h aging of bean cotyledon mitochondria isolated in five buffers. Assay media was 0.3 M in mannitol, 8 mM in succinate, 50 mM in TES, 4 mM in MgCl_2 , and 2.5 mM in KH_2PO_4 , and contained 0.002 μmoles of cytochrome c and 1.50 μmoles of ADP. Final volume 3.2 ml (pH 7.2).

requires this sharp break, and where it was not obtained ADP/O and RC were not calculated.

The ADP/O values obtained with the buffers are given in Table A-2. The differences obtained with the various buffers, although small, were consistent throughout all the trials. The ADP/O values obtained on the three successive additions of ADP to mitochondria in phosphate buffer were quite varied. However, it was established that they were always among the lowest obtained.

Use of HEPES resulted in the greatest decrease in ADP/O value after the aging period, while the other buffers were intermediate between phosphate and HEPES; aging mitochondria in phosphate did not significantly lower the ADP/O values. The transition from state 3 to state 4 became less obvious after aging in all buffers except TES. The lack of a sharp breaking point observed with fresh mitochondria in Tris buffer after the exhaustion of the third addition of ADP, was found for all ADP additions to mitochondria aged in Tris. Lack of sharp cut off point indicates the ATP synthesized is being recycled and suggests ATPase activity, as does a decrease in RC value on successive ADP additions (Wiskich, 1966).

The RC values for the various buffers are given in Table A-3. Lowering of RC on aging was more pronounced in HEPES, Tris, and Tricine buffers, while TES showed the best ADP control, and Tris the worst both before and after aging. Mitochondria in Tricine or HEPES had similar RC values before and after aging. Mitochondria aged in phosphate did not lose their ability to show three successive ADP control curves as they did when aged in Tricine and HEPES. In general,

Table A-2. Effect of various buffers on the efficiency of oxidative phosphorylation of isolated bean cotyledon mitochondria before and after a 2 h aging period.

ADP/O ratios	Buffer				
	Phosphate	Tris	Tricine	TES	HEPES
Initial	0.75	0.84	0.82	0.82	0.87
ADP/O	0.94	0.70	0.77	0.89	0.84
ratio	0.77	—	0.75	0.89	0.78
	(0.78)	(—)	(0.78)	(0.87)	(0.83)
ADP/O	0.90	—	0.68	0.89	0.68
ratio	0.80	—	0.80	0.86	0.68
after 2 h	0.77	—	—	0.82	—
aging	—	(—)	(—)	(0.86)	(—)

Three additions of ADP (0.150 μ moles each) were made before oxygen exhaustion and three ADP/O values were calculated. No values are given when results could not be calculated (—). Values in parentheses are averages. The mitochondria were aged for 2 h in the dark at 0 C. Assay medium: 0.3 M mannitol, 8 mM succinate, 4 mM MgCl_2 , 2.5 mM KH_2PO_4 , 50 mM buffer, pH 7.2. Final volume was 3.2 ml.

Table A-3. Respiratory control values of bean cotyledon mitochondria isolated in different buffers.

Buffer	RC before aging			RC after 2 h aging		
Phosphate	1.3	1.3	1.3	1.2	1.2	1.2
Tris	1.4	1.3	-	-	-	-
Tricine	1.4	1.3	1.4	1.3	1.3	-
TES	1.5	1.5	1.5	1.4	1.3	1.4
HEPES	1.4	1.3	1.4	1.2	1.2	-

RC values were calculated by division of state 3 respiration rate by state 4 respiration rate after each of three ADP additions (0.150 μ moles each).
 Assay medium: 0.3 M mannitol, 8 mM succinate, 4 mM MgCl_2 , 2.5 mM KH_2PO_4 , 50 mM buffer, pH 7.2.
 Final volume 3.2 ml.

the decreases in RC obtained on aging the mitochondria in the buffers are consistent with the decreases in respiration rate (Figure A-1 and Table A-3), and ADP/O values (Table A-2). The cotyledons used in these experiments were from 9 day old seedlings but only slightly better ADP/O and RC values could be obtained from younger tissue. It is likely then that the low values obtained for these two parameters are characteristic of senescent bean cotyledon mitochondria.

5. Effect of Buffer Purity

Ordinarily, little attention is given to the purity of compounds used as buffers. Different lot numbers of commercial TES were noted to have nitrogen analyses of 6.2% and 6.5% (theoretical is 6.12%) and this relatively large variation prompted us to investigate the effect of purity of TES.

The data in Table A-4 were obtained by use of impure TES buffer; the compound was synthesized as outlined in Methods and recrystallized only once. In comparing the data of Table A-4 with that of Table A-2 and A-3 and Figure A-1, only relative values are useful, as absolute values depend on the cotyledons from which the mitochondria were obtained. Although only the data for Tricine and TES are necessary for the comparison, phosphate and Tris are included for completeness. Tricine was superior to impure TES in both respiration rate and RC. This is in direct contrast with the results in Table A-3 and Figure A-1, where pure TES (synthesized and 4 X recrystallized) was used. The impure TES, however, did not affect the ADP/O values. Our experiences showed that impurities in zwitterionic compounds resulted in a large lowering of melting point (mp) and that

Table A-4. Three respiratory parameters of bean cotyledon mitochondria; effects of use of impure TES buffer.

Buffer	Relative respiration rate	ADP/O value	RC value
Phosphate	0.7	0.89	1.6
Tris	0.5	0.84, 0.72	1.5, 1.4
Tricine	1.2	0.95	1.9
TES (impure)	1.0	1.00	1.7

After synthesis, TES was recrystallized only once instead of the usual four times. ADP/O and RC ratios are an average of three values, obtained on three successive additions of ADP, except for Tris where only two calculations were possible and both values are given. Assay medium: 0.3 M mannitol, 8 mM succinate, 4 mM MgCl_2 , 2.5 mM KH_2PO_4 , 50 mM buffer, pH 7.2. Final volume 3.2 ml.

constancy of mp after successive recrystallizations was a good criterion of purity. A number of recrystallizations were necessary to obtain a constant mp for TES, and commercially available TES (mp 213 C) was found to have a mp almost 10 C below that of synthesized TES (4 X recrystallized). This indeed suggests an impurity in the commercial TES and it was found that the latter could not match the performance of the former. In addition, commercial TES would not dissolve completely in the grind, wash, and assay media and this again suggested the presence of impurities.

Childress and Sacktor (1966) have recently reported adverse effects from use of an impure Tris buffer. Measuring pyruvate oxidation with mitochondria from blowfly flight muscle, they found that impurities in Tris magnified both mitochondrial swelling and an inhibition of pyruvate oxidation that occurred in pure Tris. In addition, they found that shelf-stored Tris decomposed and gave poor results.

6. Effects of Other Assay Medium Components on Buffer pKa Values

The pKa values (Table A-5) of the buffers alone and in solutions containing all the components of the assay medium were determined. Tris showed an increase and phosphate a decrease in pKa value when measured in the assay medium. These differences indicated a possible interaction of the buffer with the other components of the medium.

E. Discussion

The consistently poor yields of mitochondria obtained with Tris buffer suggests that the buffer may initiate a severe clumping that causes a majority of the mitochondria to be centrifuged out in

Table A-5. Effects of assay medium components on buffer pKa values.

Buffer	pKa 0.05 M solution at 24 C	pKa of assay medium at 24 C	Change in pKa
Phosphate	6.87	6.65	-0.22
Tris	8.12	8.20	+0.08
Tricine	8.04	8.01	-
TES	7.46	7.48	-
HEPES	7.43	7.45	-

The pKa values represent pKa^2 for phosphate, pKa for Tris and pKa (protein lost) for Tricine, TES, and HEPES. Assay medium components were: mannitol (0.3 M), MgCl_2 (4 mM), succinate (8 mM), KH_2PO_4 (2.5 mM), and buffer (50 mM).

the first spin. Clumped mitochondria demonstrate poor control characteristics, and this may, at least partially, explain the poor performance of Tris. The use of phosphate buffer resulted in the highest yield of mitochondria, followed by TES and HEPES and then by Tricine and finally by Tris.

The faster respiration rate of mitochondria in TES, Tricine and HEPES buffers is obviously not a result of uncoupling, as these are the same buffers that showed the best RC. The very low rate of respiration obtained with mitochondria aged in Tris suggests that the action is on the electron transport chain and not the phosphorylation mechanism. These mitochondria showed no measureable RC and hence phosphorylation was not controlling respiration.

Acting in a manner similar to fatty acids (Eichel, 1960), the buffers might cause a lowering of mitochondrial membrane surface tension, resulting in leakage of essential respiratory components such as cytochrome c. The data in Table A-1 may enable an estimation of the degree of leakage of easily lost electron transport chain components; from this an evaluation of membrane permeability is possible. Leakage through swelling could account for the variation in cytochrome c response shown by the mitochondria isolated in Tricine, TES, and HEPES buffers.

Certain of these new buffers have proved very useful for measuring photophosphorylation (Winget, Izawa, and Good, 1965) and CO₂ fixation by isolated chloroplasts (Bucke, Baldry, and Walker, 1967; and Jensen and Bassham, 1966). Using HEPES buffer Jensen and Bassham (1966) obtained the highest efficiency of CO₂ fixation, with isolated

chloroplasts, that had been reported to that time. This buffer may minimize the leakage of essential components known to cause poor in vitro efficiencies (Whatley et al., 1956; and Smillie and Fuller, 1959); our data on cytochrome c loss from mitochondria isolated in HEPES buffer (Table A-1) also suggest this.

Uncoupling of phosphorylation from respiration was obvious with mitochondria after aging in all buffers. Only two of three ADP/O and RC values could be calculated for Tris before aging and none afterwards, while mitochondria aged in Tricine and HEPES, behaved similarly to fresh mitochondria in Tris buffer (Table A-2).

The reason for this decrease in oxidative phosphorylation on aging is unknown, but there is now good evidence that activation of phospholipases, which break down phospholipids into fatty acids and lysophosphatides, is associated with the aging of mitochondria (Estrada-O, Carabez, and Gabeza, 1966). The detergent activity of the product produces structural changes in the mitochondrial membrane with concomitant release of enzymes. It is possible that a buffer might accelerate this effect by unknown means.

The present experiments comparing the respiratory parameters of mitochondria isolated in various buffers, not only allow an evaluation of the buffers but also provide information that may help in ascertaining the mechanisms whereby buffers cause their deleterious effects.

It should be emphasized that the results reported here were obtained by use of only one system, and while there is no indication that the results cannot be generalized and applied to other systems, this

should be done with caution. For example, Frackowiak, Bryla, and Kaniuga (1967) reported similar results to those of Figure A-1 for pea seedling mitochondria, but the new buffers showed no advantages over the conventional Tris and phosphate when rat liver mitochondria were the experimental material.

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B29907